Universidade de Aveiro
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Carolina Castanheiro Frazão

Non-selective beta-blockers as potential coadjutants for prostate cancer treatment

Beta-bloqueadores não seletivos como potenciais coadjuvantes para o tratamento do cancro da próstata

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Marcelino Miguel Guedes de Jesus Oliveira, Investigador Auxiliar do Departamento de Biologia da Universidade de Aveiro e da Doutora Maria de Lourdes Pereira, Professora Associada com Agregação do Departamento de Ciências Médicas da Universidade de Aveiro.

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palavras-chave

Beta-Bloqueadores, Linhas Celulares, Cancro da Próstata, Viabilidade Celular, Combinações de Tratamentos

resumo

O cancro de próstata é o terceiro tipo de cancro mais diagnosticado em todo o mundo e a quinta causa de morte relacionada com cancro em homens. Atualmente, os tratamentos disponíveis nem sempre são eficazes. Por esse motivo, torna-se imprescindível explorar novas formas de tratamento que podem incluir o uso de medicamentos, já disponíveis clinicamente, para o tratamento de outras doenças, como os β-bloqueadores. O presente estudo teve como objetivo explorar os efeitos de vários β-bloqueadores e medicamentos tipicamente usados no tratamento do cancro da próstata em linhas celulares deste cancro (22Rv1, LNCaP e PC3) e numa linha celular de tecido normal da próstata (PNT-2). Para este efeito, as linhas selecionadas foram expostas, até 72 h, a uma gama de concentrações (10-250 ou 0,1-100 μM) de β-bloqueadores não seletivos (propranolol e carvedilol), bloqueadores β1 (atenolol e metoprolol), um medicamento citostático usado em quimioterapia (cisplatina) e um medicamento que bloqueia o recetor androgénico, usado em terapia hormonal (flutamida), sendo a viabilidade celular avaliada após o período de exposição, com recurso ao 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Os resultados obtidos revelam que os β-bloqueadores não seletivos selecionados bem como os citostáticos apresentaram efeito citotóxico em todas as linhas celulares, enquanto os β1-bloqueadores testados não alteraram significativamente a viabilidade celular, na gama de concentrações testada. Das linhas celulares testadas, 22Rv1 foi a linha mais sensível ao propranolol, carvedilol e cisplatina sendo a linha PC3 a que apresentou menor sensibilidade aos fármacos testados. Com base nos resultados das exposições individuais, as linhas 22Rv1 (a linha mais sensível), PC3 (a linha menos sensível) e PNT-2 (a linha celular normal) foram submetidas a uma exposição combinada de propranolol e cisplatina ou propranolol e flutamida ou cisplatina e flutamida, de forma a avaliar a potencial interação do β-bloqueador com os compostos citostáticos, de forma a avaliar a sua potencial aplicação em tratamentos combinados. De uma forma geral, as exposições combinadas revelaram interações dependentes da concentração entre os fármacos citostáticos e o propranolol, sendo dentro das combinações testadas, a de propranolol com a cisplatina a mais promissora para o tratamento deste cancro, abrindo novas perspetivas para o combate desta patologia.

keywords Beta-Blockers, Cell Lines, Prostate Cancer, Cell Viability, Combined Treatments

abstract

Prostate cancer is the third most diagnosed type of cancer worldwide and the fifth leading cause of death in men. Currently, available treatments are not always effective. For this reason, new forms of treatment need to be explored, which may include the use of medications, already clinically available, for the treatment of other diseases, such as β-blockers. The present study aimed to explore the effects of various β-blockers and pharmaceuticals typically used in the treatment of prostate cancer on prostate cancer cell lines (22Rv1, LNCaP and PC3) and on a normal prostate tissue cell line (PNT-2). For this purpose, selected lines were exposed, up to 72 h, to a range of concentrations (10-250 or 0.1-100 μM) of non-selective β-blockers (propranolol and carvedilol), β1 blockers (metoprolol and atenolol), a cytostatic drug used in chemotherapy (cisplatin) and a drug that blocks the androgen receptor, used in hormonal therapy (flutamide) with cell viability assessed after the exposure period, using 3-(4,5-dimethylthiazol-2 -yl)-2,5-diphenyltetrazolium bromide (MTT). The results obtained reveal that selected non-selective β-blockers as well as cytostatic drugs showed a cytotoxic effect in all cell lines, while the β1-blockers tested did not significantly alter cell viability, in the range of concentrations tested. Of the cell lines tested, 22Rv1 was the most sensitive to propranolol, carvedilol, and cisplatin, with the PC3 line showing the lowest sensitivity to the drugs tested. Based on the results of the individual exposures, the 22Rv1 (the most sensitive line), PC3 (the least sensitive line), and PNT-2 (the normal cell line) lines were subjected to a combined exposure of propranolol and cisplatin or propranolol and flutamide or cisplatin and flutamide in order to assess the potential β-blocker interaction with cytostatic compounds, in order to assess their potential application in combined treatments. Overall, the combined exposures revealed concentration-dependent interactions between cytostatic drugs and propranolol. Among the tested combinations, propranolol with cisplatin is the most promising for the treatment of this cancer.

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1. Introduction

There has been a considerable technological development over the last years that allowed the improvement in human life quality and health services. These improvements contributed to an increase of human life span. However, despite this improvement, the current human lifestyle (e.g., quality of diet, exposure to chemicals through food, air, and water) has promoted an increase in cancer incidence.

1.1. Prostate Cancer

Prostate cancer, according to the data provided by the Global Cancer Observatory of 2020, is in the top five in terms of both incidence and mortality rates, worldwide (Observatory, 2020). The prostate is a small gland of the male reproductive system, located in the pelvis, between the penis and the bladder. The prostate gland, that during gestation starts to form from the urogenital sinus (UGS), has its development dependent on androgens, in particular dihydrotestosterone (DHT). After birth, the prostate maintains its constituents, up until the beginning of puberty, when the gland starts to change morphologically into the adult phenotype (Hammerich et al., 2008). The prostate helps in the control of urine output from the bladder, produces the prostatic fluid, a component of the seminal fluid that is rich in several proteins (e.g., prostatic acid phosphatase (Lilja & Abrahamsson, 1988)), essential for nourishing and protection of the viability of the sperm and that metabolizes testosterone to a more potent androgen, DHT, which influences the hypothalamic-pituitary axis (Huggins, 1945; Kumar & Majumder, 1995; Rizzo et al., 2005).The prostate can be divided into four major zones (McNeal, 1981): peripheral zone (that contains most of the prostatic glandular tissue and is where diseases such as carcinoma and chronic prostatitis usually develop), central zone (surrounding the ejaculatory ducts), transitional zone (that surrounds the prostatic urethra and is related to the development of benign prostatic hyperplasia) and, anterior fibromuscular stroma (constituted mostly of striated and smooth muscle, playing a role on the voluntary and involuntary sphincter functions (Hammerich et al., 2008; Oh et al., 2003)). Histologically, the prostate has two cell layers, an epithelial secretory cell layer, androgen-dependent, for growth, that produces proteins, and, an underlying basal cell layer, growth androgen-independent, that contains the stem cell population of the epithelial prostate cells (Oh et al.,

2003; Rizzo et al., 2005). The androgens are essential for the maintenance of the prostate structure and function.

Like any other cancer, prostate cancer (PCa) begins when abnormal or damaged cells start to grow without control, which may occur due to several irreversible oncogenic mutations. These cells can originate a tumour. If the tumour does not have the ability to invade neighbouring tissues or metastasize is considered benign tumour. However, if the tumour has the ability to spread into other tissues nearby or far away from the original tissue, in a process called metastasis, it is considered a malignant tumour, on the other hand, can. The ability of invasion and the requirements of nutrients needed for cell survival are facilitated by the ability to develop a capillary network (process called angiogenesis) (Institute, 2021).

Most prostate cancers are considered adenocarcinomas (Abate-Shen & Shen, 2000). They originate from the gland cells responsible for making the prostatic fluid that is added to the semen. However, in rare cases the detected cancer can be a small cell carcinoma, a neuroendocrine tumour, a transitional cell carcinoma or a sarcoma (The American Cancer Society, 2019b).

One of the major challenges associated with PCa treatment is related with the difficulty of diagnose in early stages, as it is asymptomatic. Symptoms only begin to appear in more advanced stages, such as fatigue, bone pain, difficulty urinating and weight loss (Smith et al., 1999; Victorson et al., 2011). The only signal present at early stages may be a slight increase of the Prostate-Specific Antigen (PSA) (Miller et al., 2003).

1.2.1. Epidemiology, Risk Factors and Diagnosis

According to the data provided by the Global Cancer Observatory of 2020 (Observatory, 2020) prostate cancer is in the top five in terms of both incidence and mortality rates, worldwide. In 2020, 1 414 259 new cases and 375 304 deaths related to prostate cancer were reported worldwide (Observatory, 2020).

Aging is a significant risk factor for prostate cancer with most of detectable cancers being diagnosed after the age of 65 (Patel & Klein, 2009). Diet and environment may also play an important role in the prostate carcinogenesis, especially when a person immigrates from a country with low incidence to high incidence to prostate cancer (e.g., from Japan to United States) (Carter et al., 1990). For example, the western lifestyle, where there is a high intake of polyunsaturated fats, can induce hormonal alterations or elevate

oxidative stress levels (Bostwick et al., 2004; Crawford, 2003; Grönberg, 2003) increasing the risks of disease. Androgens levels may also affect the risk of prostate cancer. Endogenous (e.g., genetics) and exogenous factors (e.g., exposure to endocrine disrupting chemicals) that are able to modulate the levels of androgens, may also alter the risk of prostate cancer as this disease is androgen-dependent. Ethnicity has also been considered as a potential risk factor. A higher risk to develop prostate cancer has been reported for African-American men than for Caucasian-American men. These differences may be due to dietary and genetic differences but, they may also reflect the difference in the access to medical care (Bostwick et al., 2004; Patel & Klein, 2009).

The detection of PCa is supported by the measurement of Prostate-Specific Antigen (PSA) levels, a glycoprotein, produced only in the prostate gland (Herrala et al., 2001). In healthy men, blood PSA levels are low, usually under 4 ng/mL of blood (The American Cancer Society, 2019a). However, in the early stages of cancer development, disruption of the prostate epithelium promotes increased levels of PSA in the blood vessels, which can be used as a biomarker of PCa (Drake et al., 2015). Despite being used to signal PCa, PSA levels may also serve as a warning signal for other diseases such as prostatitis (inflammation of the prostate). Factors like age, may modulate their levels (Loeb et al., 2006). Thus, although it is a sensitive organ-specific biomarker, PSA is not specific for cancer (Munteanu et al., 2020). In this sense, precise diagnosis requires the use of updated serum-based biomarkers, which may be analysed using indexes (e.g., Prostate Health Index (PHI) and four kallikrein (4K)) or urine-based (e.g., Prostate Cancer Antigen 3 (PCA3) and Hoxc6/dlx1). PHI is a mathematical formula that combines total PSA, free PSA and pPSA (inactive precursor form of PSA). 4K combines four kallikrein proteins, such as total PSA, free PSA, intact PSA and human kallikrein 2. PCA3 and *hoxc6/dlx1* genes are overexpressed in prostate cancer (Tan et al., 2019; Velonas et al., 2013). Nanotechnologies have allowed methodological advances for a better detection of PSA levels in prostate cancer (Akl et al., 2020; Farschi et al., 2020). The digital rectal examination (DRE), is among the common approaches used to detect prostate problems, that can perceive nodules or lumps while assessing the prostate size (Waldron & Chowdhury, 2020). Occasionally, sampling of prostate tissue (by transrectal or transperineal ultrasound-guided biopsy (Waldron & Chowdhury, 2020)) may be required to confirm, or not, the diagnosis. Over the years, new imaging techniques have emerged to improve diagnostic accuracy and sensitivity such as, multiparametric magnetic resonance imaging (mpMRI), whole-body diffusion-weighted MRI, nano-MRI and positron emission tomography/computed

tomography (PET/CT) using several radiotracers like ^{68}Ga prostate-specific membrane antigen (PSMA), ^{11}C-Choline and ^{18}F-fluciclovine (Fennessy et al., 2020; Gupta et al., 2020; Scholte et al., 2020). ^{68}Ga-PSMA can detect the expression of PSMA in the cells (Scholte et al., 2020). ^{11}C-Choline takes advantage of the high metabolic need of cancer for choline, for the formation of the membrane and cellular signalling (Reske et al., 2008). ^{18}F-fluciclovine is used for the detection of metastatic disease of patients who already had treatment and have an elevation of PSA levels (Ren et al., 2016). It uses the upregulated activity of alanine, serine, cysteine transporter 2 (ASCT 2) and L-type amino acid transporter 1 (LAT 1) (Oka et al., 2012).

Nonetheless, even though the PSA biomarker has sensitivity limitations, it is the most used in the diagnosis and surveillance of prostate cancer. Thus, there is the need to find new biomarkers able to signal early and accurately this pathology and able to identify high-risk individuals (Chung et al., 2020; Intasqui et al., 2018; Velonas et al., 2013). In this sense molecular biomarkers have been the focus of intense research in the recent years. Since the prostate cancer is predominantly androgen-dependent (Campbell et al., 2020; Teo et al., 2019), the androgen receptor has been one of the foci of investigation, especially the androgen receptor splice variant 7 (AR- V7) that even without a ligand-binding domain is able to remain active and confer resistance to the androgen receptor blocker enzalutamide and the anti-androgen abiratone (Teo et al., 2019). Altered expressions of genes associated with E-twenty-six (ETS) family, tumour protein p53 (TP53) and Phosphatase and Tensin Homolog (PTEN) in patients with prostate cancer, aberrations in the phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (AKT) pathway, DNA repair pathway (in *brca1/brca2*, *atm* and *chek2* genes), cyclin dependent kinases (CDK) inhibitors, Wingless-related integration site (Wnt) pathway and microsatellite instability have been reported (Chung et al., 2020; Fu & Chi, 2018; Mohler et al., 2019; Teo et al., 2019) and, led to its consideration as potential biomarkers.

1.2.2. Treatments

1.2.2.1. Active Surveillance

Active surveillance means that the patient will be closely monitored for the progression or lack of the cancer to prevent unnecessary treatment. This approach involves regular visits to the doctor to do PSA blood test, biopsies, DRE and/or MRI. A progression of the cancer supports the need of a new treatment (Dahabreh et al., 2012; Waldron & Chowdhury, 2020).

1.2.2.2. Radical Prostatectomy and Radiation Therapy

In the clinical guidelines, radiotherapy, as well as radical prostatectomy with or without hormone therapy, are considered primary treatment options for patients with localized prostate cancer (Mohler et al., 2019; Sanda et al., 2017).

The radical prostatectomy is the oldest treatment used for prostate cancer, often considered when the cancer has not spread beyond the prostate gland. This approach involves the removal of the entire prostate gland as well as the seminal vesicles and the lymph nodes from the pelvis (pelvic lymphadenectomy). The patient may have a complete surgical resection of the cancer, leaving normal periprostatic tissues intact and avoiding urinary incontinence and erectile dysfunction (Sebesta & Anderson, 2018; Teo et al., 2019).

The radiation therapy uses high-energy rays or particles to kill cancer cells. It can be delivered in two ways: as an external beam therapy (EBRT), where usually the whole gland is irradiated (as the machine is outside the body the radiation needs to pass through healthy tissue to reach its target); in another approach, the radioactive material, usually in higher concentrations, is directly delivered to the problematic area (e.g., brachytherapy) with or without permanent implantation of radioactive seeds. Brachytherapy can be used as a monotherapy or in combination with EBRT, surgery and/or chemotherapy (Tanderup et al., 2017). With the advances in technology, there are more recent versions of EBRT, such as three-dimensional conformal or stereotactic radiation therapy, intensity modulated radiation therapy and heavy particle radiotherapy (Evans, 2018). However, brachytherapy as a monotherapy is still predominant, showing a higher efficiency, lower cost and fewer side effects than EBRT (Tanderup et al., 2017; Zaorsky et al., 2017). Radium-223 (an alpha particle emitter that causes DNA double strand breaks) (Parker et al., 2013) is an example

of the radioactive seeds used in brachytherapy, especially for the treatment of bone metastases, since it targets their microenvironment.

1.2.2.3. Focal Therapy

Focal therapy or ablative therapy emerged as an option for patients with localized prostate cancer who do not wish to suffer the severe side effects of radical procedures (e.g. radical prostatectomy), but still want some treatment in opposite to the active surveillance (Winoker et al., 2018). These minimally invasive therapies are easy to achieve and, due to their nature, can be performed multiple times. Ablative therapies aim to destroy cancer cells in a minimally invasive manner, using several energy resources, without damaging adjacent structures (Ramsay et al., 2015). The whole gland, half the gland or a specific area can be treated, making use of the information from the MRI and biopsies (Evans, 2018).

High-intensity focused ultrasound uses high-energy ultrasound waves (0,8 – 3,5 MHz) to induce coagulative necrosis at a specific target by heating the tissue to or above 60 °C (Ramsay et al., 2015). Focal laser ablation needs a laser fibre directly in contact with the cancer, with the help of real-time MRI, to convert the laser energy to heat (from 42 ºC to above 60 ºC (Ahdoot et al., 2019; Winoker et al., 2018)) leading to coagulative necrosis (protein denaturation and irreversible tissue damage). Vascular targeted photodynamic therapy requires a photosensitizer (a light-sensitive agent) that can be activated by visible light (732-763 nm) from an optical fibre placed directly on the target in the presence of oxygen. This activation leads to the formation of reactive oxygen species, creating vascular necrosis in the tumour blood vessels (Evans, 2018; Ramsay et al., 2015). In irreversible electroporation, a probe is placed in the target tissue under ultrasound or MRI guidance. The probe releases high-voltage bursts of electric current which causes the formation of pores in cells, leading to cell death (Ahdoot et al., 2019; Davalos et al., 2005; Winoker et al., 2018). In contrast to the previous therapies that rely on higher temperatures, cryotherapy drops the temperature of the target tissue to -40 °C, by introduction of two gases, one for the freezing phase (argon gas) and the other for the active thawing phase (helium gas).The first phase induces extracellular water freezing, creating intracellular dehydration and osmotic stress, while the second phase leads to intracellular ice crystal formation, which consequently leads to irreversible cell disruption and apoptosis (Ahdoot et al., 2019; Evans, 2018; Winoker et al., 2018).

Table 1: Types of ablative therapies and their reported main side effects. Source: Ramsay et al. (2015).

Ablative Therapy	Principal Side Effects
High-Intensity Focused Ultrasound	Acute urinary retention Erectile dysfunction Urethral structure Rectourethral fistula Pelvic pain
Focal Laser Ablation	Transient perineal discomfort Haematuria
Vascular Targeted Photodynamic Therapy	Phototoxicity Skin photosensitization Erectile dysfunction Urethral damage Rectourethral fistula
Cryotherapy	Erectile dysfunction Urinary incontinence Urethral sloughing Rectal injury Rectourethral fistula

1.2.2.4. Hormone Therapy

Testosterone, the main circulating androgen in men, is produced by Leydig cells under the control of the hypothalamic-pituitary axis. In the prostate, testosterone is metabolized by 5α-reductase to dihydrotestosterone, a ligand for the androgen receptor (AR). Some variants of this receptor (e.g., AR-V7) may be responsible for the resistance to hormone therapy (Abraham & Staffurth, 2020). Since prostate cancer is considered an androgen dependent cancer (Huggins & Hodges, 1941), hormone therapy or androgen deprivation therapy (ADT) is a standard treatment for this cancer (Abraham & Staffurth, 2020).

ADT aims to achieve castration levels of testosterone in the circulation by reducing testosterone secretion or by inhibiting the action of the androgen receptors. Usually, the drugs used for ADT are anti-androgen and gonadotropin-releasing hormone (GnRH) agonists or antagonists (Table 2) (Wu et al., 2020). They can also be combined to form a complete androgen blockade (CAB) (Iguchi et al., 2019). An anti-androgen drug can block the production of testosterone by inhibiting the 17 α-hydroxylase/C17,20-lyase (CYP17) enzyme (Marandino et al., 2020), lowering androgen levels in testes, adrenal glands, and prostate cancer cells (Raghavan, 2018).

GnRH agonists use the negative feedback loop of the hypothalamic-pituitary axis to reduce androgen levels. In the beginning the levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone rise due to stimulation. However, after a while, the negative feedback loop is activated, and there is a downregulation of the GnRH receptor leading to a reduction in the levels of the three hormones. GnRH antagonists, on the other hand, can immediately suppress the production of FSH, LH, and testosterone (Campbell et al., 2020).

The androgen receptor blockade competes with testosterone for AR binding. AR translocation to the nucleus can also be blocked as well as the recruitment of co-factors needed for this receptor and AR binding to DNA (Marandino et al., 2020). With this approach, circulating testosterone levels are not decreased (Campbell et al., 2020).

When cancer is in an androgen-deprived environment for a long period of time, it can lead to splice variants in the androgen receptor, like the androgen receptor splice variant 7 (AR-V7), which lacks the ligand-binding end. Despite the low levels of androgens, the receptor remains active as a transcription factor in a ligand-independent manner and the hormonal agents that act on these receptors no longer have effect on them (e.g., enzalutamide) (Antonarakis et al., 2014; Raghavan, 2018).

Table 2: Drugs commonly used in hormone therapy and their modes of action. Source: Abraham & Staffurth (2020). GnRH: gonadotropin-releasing hormone, FSH: follicle-stimulating hormone, LH: luteinizing hormone and AR: androgen receptor.

Type of Drug	Drug	Mechanism of Action
GnRH Agonist	Leuprorelin	Reduced pituitary production of LH and FSH (Initial surge of testosterone)
	Goserelin	
	Triptorelin	
	Histrelin	
GnRH Antagonist	Degarelix	Reduced pituitary production of LH and FSH (Without initial surge of testosterone)
	Relugolix	
Androgen Synthesis Inhibition (Anti-Androgen)	Abiraterone Acetate	Reduced androgen production
Androgen Receptor Blockade	**First Generation**	Competitive AR inhibition and block of AR translocation to the nucleus
	Flutamide (also, an anti-androgen)	
	Nilutamide	
	Bicalutamide (also, an anti-androgen)	
	Second Generation	
	Enzalutamide	
	Apalutamide	
	Darolutamide	

1.2.2.5. Chemotherapy

Platinum-based chemotherapeutics have been used for a long time to treat multiple cancers. Cisplatin, since its discovery, has been widely used in the treatment of multiple solid cancers, such as testicular, ovarian, and colorectal cancer (Yimit et al., 2019). However, because of the severe side effects for the patients (Table 3), this chemotherapeutic drug has limited use. Furthermore, the cancer may have, or develop, a resistance to platinum-based drugs, further limiting their use (Jung & Lippard, 2007). The resistance usually happens as a result of cellular adaptations, which include low levels of drug uptake, inactivation by several molecules (e.g., glutathione and metallothionein), improved control of DNA damage repair, or increased damage tolerance (Wang & Lippard, 2005; Yimit et al., 2019). Cisplatin can have effects on several cell components but DNA is the primary target, with the platinum molecule binding to DNA purines and forming DNA adducts, disrupting cell processes such as DNA replication and transcription (Basu & Krishnamurthy, 2010). In response, the cell can activate several repair mechanisms, the main one being the nucleotide excision repair (NER) pathway, responsible for the removal of DNA adducts. The NER pathway has two sub-pathways: transcription-coupled repair (TCR) and global genomic repair (GGR), that help in the recognition and repair of specific parts of damaged DNA. However, these DNA adducts cannot be repaired most of the time, leading ultimately to cellular apoptosis (Jung & Lippard, 2007; Wang & Lippard, 2005).

Other families of non-platinum based anti-neoplastic drugs have been considered for the treatment of prostate cancer, including, taxane. This anti-neoplastic family includes several compounds like paclitaxel, docetaxel and cabazitaxel, which inhibit microtubular depolymerization. This effect is caused by their binding to β-tubulin, leading to microtubule polymerization, even without cofactor proteins and guanosine triphosphate. Once assembled, microtubules cannot dissociate even in the presence of calcium ions or at 4 ºC, leading to apoptosis, as the cell cycle will be halted in the G2 and M phase. These cells also exhibit phosphorylation of the apoptotic marker Bcl-2 (Imran et al., 2020; Pienta, 2001). Docetaxel is currently the most used chemotherapeutic drug for the treatment of prostate cancer. When the cancer acquires resistance to docetaxel, it can be replaced by cabazitaxel (Teo et al., 2019). The main mechanism of cell resistance to docetaxel involves the increased expression of P-glycoprotein, responsible for the transport of the docetaxel to the exterior of the cell. The low affinity between cabazitaxel and P-glycoprotein increases the potential effect on cancer cells (Mizokami et al., 2017). Like cisplatin, treatments with taxanes may also induce severe side effects shown, in Table 3.

Table 3: Chemotherapeutic drugs commonly used for prostate cancer treatment and their modes of action. Sources: Imran et al. (2020); Teo et al. (2019). NER: nucleotide excision repair, TCR: transcription-coupled repair and GGR: global genomic repair.

Chemotherapeutic Drug	Mechanism of Action	Repair Pathways Activated	Severe Side Effects
Cisplatin	DNA Adducts	NER TCR GGR	Ototoxicity Peripheral Neuropathy Myelosuppression Nephrotoxicity
Docetaxel	Inhibition of microtubular depolymerization Phosphorylation of Bcl-2		Peripheral Neuropathy Stomatitis Peripheral Oedema Alopecia Nail Disorders
Cabazitaxel			Febrile Neutropenia Neutropenic Infection Diarrhea Haematuria

1.2.2.6. Immunotherapy

Immunotherapy aims to increase the strength of the patient immune system by stimulating immune cells (e.g., through vaccines) or by finding ways to counteract signals that suppress the immune system (e.g., immune checkpoint inhibitors) (Schatten, 2018).The slow and progressive nature of PCa, allows the body to generate an antitumor immune response. However, this cancer can escape this immune response by inhibiting the presentation of the tumour antigen (Drake et al., 2006), secreting cytokines, that can

suppress the immune system or inducing apoptosis in immune cells (Bilusic et al., 2017; Drake et al., 2006; Schatten, 2018).

Sipuleucel-T was the first autologous vaccine approved by the United States Food Drug Administration (FDA) for the treatment of metastatic castration-resistant prostate cancer (mCRPC) (Schatten, 2018). This vaccine is derived from peripheral dendritic cells collected from the patient, stimulated, and activated with PA2024, a recombinant fusion protein which includes a prostate antigen – prostatic acid phosphatase (PAP) – linked to a granulocyte-macrophage colony-stimulated factor (GM-CSF). These cells are then infused back into the patient (Bilusic et al., 2017; Chakravarty et al., 2020; Kantoff et al., 2010; Silva et al., 2020).

Examples of other vaccines, not yet FDA approved, include PROSTVAC, is composed of a heterologous prime-boost regimen using two different live poxviral-based vectors: a recombinant vaccinia virus, as the prime, and a recombinant fowlpox virus, as the boost. Both vectors contain genes for PSA, and three immune-enhancing costimulatory molecules: B-lymphocyte activation antigen B7.1 (B7.1), intercellular adhesion molecule-1 (ICAM-1), and leukocyte function-associated antigen-3 (LFA-3), collectively they form PSA-TRICOM, and DCVAC/PCa, a vaccine composed of mature dendritic cells that have been exposed to killed human PCa cells (LNCaP) (Bilusic et al., 2017; Gulley et al., 2019; Silva et al., 2020).

Immunotherapy can also involve immune checkpoint inhibitors that target the immunosuppressive pathways originating from tumour cells, such as the cytotoxic T lymphocyte antigen 4 (CTLA4) (capable of inhibiting T cell activation) and the programmed cell death 1 ligand 1 (PD-L1 or B7H1) (that can inhibit the antitumoral response) (Schatten, 2018). Ipilimumab, a monoclonal antibody, can block the CTLA4 leading to T-cell activation, proliferation and finally cause cancer cell death (Silva et al., 2020). Nivolumab and pembrolizumab are also monoclonal antibodies, but they target PD-L1. These antibodies can be used alone or in combination (Schatten, 2018).

Finally, chimeric antigen receptor (CAR) T-cell therapy was developed to combine the functions of T-cells and the properties of antibodies (Chakravarty et al., 2020). CAR-T cells are autologous T lymphocytes, genetically modified to express a receptor specific to an antigen (e.g., PAP), derived from the antibody, and have several signalling domains, to activate T-cells more easily before being reinfused back to the patient. In this approach, the patient's own immune cells will be able to easily detect and destroy cancer cells (Chakravarty et al., 2020; Fay & Graff, 2020; Schatten, 2018; Silva et al., 2020).

1.2.2.7. Nanotherapeutics

All drugs previous described have limitations after administration (e.g., uneven, or unwanted distribution, fast clearance, inability of reaching the tumour microenvironment or toxicity). Nanotherapeutics aim to overcome such challenges by delivering the active molecules specifically to the tumour, provoking minimal damage to healthy tissues (Katsogiannou et al., 2011; Ruoslahti, 2017; Thakkar et al., 2020). The drug delivery to the tumour can be achieved through active or passive targeting. The active targeting takes advantage of receptors expressed on tumour cells, using them to connect the nanocarriers (e.g., liposomes, polymers, micelles, engineered antibodies, viral nanoparticles, dendrimers, metal nanoparticles or polymeric nanoparticles), that can carry several moieties (e.g., antibodies, aptamers, peptides, sugars, and other small molecules), which will release the chemotherapeutic drug or other active molecule, for example miRNA, directly into the microenvironment of the tumour. Passive targeting, on the other hand, uses the enhanced permeation and retention (EPR) effect to increase the number of active molecules in the tumour, taking advantage of the local vascular permeability and reduced lymphatic drainage. In addition, the unique conditions of the microenvironment surrounding the tumour can also be used. Due to the high metabolic needs, tumour cells use glycolysis for extra energy, creating an acidic environment. pH-sensitive nanoparticles, stable at normal physiological pH, and that disintegrate releasing their content in environments with lower pH levels, such as inside tumour cells, can also be used (Kanapathipillai et al., 2014; Katsogiannou et al., 2011; Sanna et al., 2013).

1.2.3. Adrenergic Receptors

Many cells in the body, including cancer and immune cells, have adrenergic receptors (Fumagalli et al., 2020; Servick, 2019), transmembrane glycoproteins that interact with catecholamines, norepinephrine and epinephrine. These receptors are coupled to guanine nucleotide (GTP) binding proteins (G proteins), that mediate the responses of the sympathetic nervous system. These receptors can be divided into two main groups: β- (ADRB) and α-adrenergic receptors (ADRA). ADRB can still be divided into subgroups: β-1-, β-2- and β-3-receptors (Abosamak & Shahin, n.d.; Braadland et al., 2015; Graham, 1990).

The prostate has several types of nerves, essential for its development and maintenance (Coarfa et al., 2018; Rodrigues et al., 2002), including sympathetic nerves,

responsible for the “fight or flight” response to a threat, by releasing catecholamines into the tissue. Catecholamines can increase the heart rate and blood pressure. Adrenal glands, present in the kidneys, also secrete the same hormones into the bloodstream, to reach other organs (Servick, 2019).

When prostate cancer cells form a tumour, they release neurotrophic factors to attract into the tumour nerves specifically sympathetic nerves, to release catecholamines to nearby tissues (March et al., 2020; Servick, 2019). If the tumour is at an advanced stage, it can even promote the creation of new nerves (neoneurogenesis) (Ayala et al., 2008; Magnon et al., 2013). The activation of the β-adrenergic receptors by hormones will promote their proliferation (Servick, 2019). The prostate is very rich in ADRB with ADRB2 being the principal isoform (Braadland et al., 2015; Nagmani et al., 2003). Norepinephrine can activate the receptors on endothelial cells to promote the formation of new blood vessels to deliver oxygen into the growing tumour (neoangiogenesis) (Chakroborty et al., 2009; Sarkar et al., 2013). This process will also facilitate the invasion of cancer cells into other tissues, creating metastases. Nerves can also send signals to immune cells, such as macrophages or T cells, to prevent their attack on the tumour and promote its growth (Servick, 2019). This is why most prostate cancers are found in the peripheral zone of the prostate, since it is the location of most nerves (Braadland et al., 2015).

1.2.4. Cancer Treatment, Cardiotoxicity, and β-Blockers

The traditional cancer treatments (e.g., chemotherapy) can carry major cardiovascular complications (Campbell et al., 2020; Lenneman & Sawyer, 2016). For instance, in the case of cisplatin, as it is not completely eliminated from the organism (Brouwers et al., 2008), it can cause problems in the endothelium, leading to endothelial dysfunction and adverse effects on the cardiovascular system (Lenneman & Sawyer, 2016). The major cardiovascular issues created by cisplatin are arrhythmias, hypertension, angina, coronary artery disease, cardiac ischemia, myocarditis, pericarditis, diastolic disturbances, acute myocardial infarction, thromboembolic events, and chronic heart failure (Haugnes et al., 2010; Patanè, 2014). Even though most of the reports of cardiotoxicity are from radio- and chemotherapy, there have been studies associating cardiovascular problems with hormone therapy. Hormonal therapies have several effects on the body that can contribute to cardiovascular problems, such as a change in the body composition (less lean mass and an increase in fat mass), hepatic fat accumulation, glucose metabolism, lipid metabolism

and arterial wall composition. The longer the patient is under the therapy the higher the chances leading to hypertension, strokes, arrhythmias, and myocardial infarction (Campbell et al., 2020; Levine et al., 2010; Okwuosa et al., 2021).

β-adrenergic receptor-blocking agents (β-Blockers) have been considered for cancer treatment not only because they are commonly described as cardioprotectors but also because they can block receptors that are associated with mechanisms that trigger tumorigenesis, angiogenesis and tumour metastasis (Antoni et al., 2006; Cole & Sood, 2012; Peixoto et al., 2020). β-adrenergic antagonists compete with catecholamines for the connection to the β-adrenergic receptors (do Vale et al., 2019). For this reason, they are being considered as a new potential form of cancer treatment, considering the role of catecholamines in cancer progression (Marino & Cosentino, 2013; Tang et al., 2013).

β-Blockers can be selective for a β-adrenergic receptor, such as β-1, β-2, or β-3, or be non-selective, which means they have similar affinity for β-1 and β-2(Baker et al., 2011; Fumagalli et al., 2020; Mravec et al., 2020). Since the discovery of propranolol in 1960s by Sir James Black (Black & Stephenson, 1962; Srinivasan, 2019), three generations of β-Blockers have been introduced into the clinical practice (Table 4) (Bond, 2009; Fumagalli et al., 2020).

Table 4: β-Blockers commonly used in the clinical practice. Source: Fumagalli et al. (2020).

Generation	Drug	Selectivity
First	Nadolol Penbutolol Pindolol Propranolol Timolol	Non-selective β-blockers
Second	Acebutolol Atenolol Bisoprolol Esmolol Metoprolol	β-1-blockers
Third	Carteolol Carvedilol Labetalol Bucindolol	Non-selective β-blockers
	Betaxolol Celiprolol Nebivolol	β-1-blockers

1.2. Objectives

The high incidence of cancer worldwide and limited efficiency of available treatments make the scientific research for more efficient treatment approaches a priority. In this sense, there has been intense research to improve the efficiency of the available cancer treatments. This dissertation aimed to provide relevant data that ultimately may improve the efficiency of PCa treatment. Thus, the hypothesis that β-blockers can decrease the proliferation rates of cancer cells was tested. The dissertation goals included the evaluation of the effects of different types of β-blockers (atenolol, carvedilol, metoprolol, and propranolol) on prostate cancer cell lines (22Rv1, LNCaP and PC3) and in a normal prostate cell line (PNT-2), alone and combined with a cytostatic drug (cisplatin and flutamide), to provide scientific data to understand if β-blockers can be used for the treatment of this cancer.

2. Materials and Methods

2.1. Chemicals

Propranolol (non-selective β-blocker, CAS 318-98-9), atenolol (β-1-blocker, CAS 29122-68-7), metoprolol (β-1-blocker, CAS 56392-17-7), carvedilol (non-selective β-blocker, CAS 72956-09-3), cisplatin (cytostatic drug, CAS 15663-27-1) and flutamide (androgen receptor blocker cytostatic drug, CAS 13311-84-7) were purchased from TCI chemicals (Belgium).

Stock solution of propranolol (2 mM), metoprolol (2 mM), and atenolol (2 mM) were prepared in ultra-pure water and filtrated, whereas carvedilol (100 mM), cisplatin (500 mM) and flutamide (500 mM) were prepared in dimethyl sulfoxide (DMSO). The stock solutions were diluted in RPMI-1640 medium to achieve the desired drug concentrations and filtrated (0.22 µm pore PES filter) to achieve sterilization. All other reagents used were analytical grade (Sigma-Aldrich, Spain).

2.2. Cell Lines

Prostate cancer 22Rv1, LNCaP and PC3 cell lines were kindly provided by Doctor João Carvalho of the Netherlands Cancer Institute and the normal prostate PNT-2 cells were kindly given by Doctor Paula Guedes Pinho, of the Faculty of Pharmacy, Porto University. 22RV1 is a human prostate carcinoma epithelial cell line derived from a xenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft (Sramkoski et al., 1999). LNCaP is derived from a metastatic lesion on the left supraclavicular lymph node of human prostatic adenocarcinoma (Horoszewicz et al., 1980). PC3 is derived from a bone metastasis of a grade IV prostatic adenocarcinoma (Kaighn et al., 1979). Both 22Rv1 and LNCaP cell lines express the androgen receptor (AR) and prostate-specific antigen (PSA) and are androgen dependent. On the other hand, PC3 does not express AR and PSA and is androgen independent. PNT-2 is a normal adult prostatic epithelial cell line, immortalised by transfection with the defective whole genome of the simian virus 40 (SV40 ori) (Berthon et al., 1995). All cell lines were maintained in RPMI-1640 medium supplemented with 2 mM de L-glutamine (Biowest, France), 10% fetal bovine serum (FBS, Gibco, USA), 100 µg/mL streptomycin, 100 U/mL penicillin G, 100 µg/ml gentamicin and 2,5 µg/ml amphotericin B

(Biowest, France). The cells were maintained at 37 ºC in a humidified incubator, supplemented with 5% of carbon dioxide. Cells were routinely cultivated in 10 cm culture dishes.

2.3. Exposure Design

2.3.1. Individual Exposure

In order to evaluate the effect of each drug on each cell line, this study assessed the cell viability (through thiazolyl blue tetrazolium bromide (MTT)). Cell lines 22Rv1, LNCaP, PC3 and PNT-2 were plated onto flat-bottom clear 96-well plates, at a cell density of $1x10^4$ cells per well, and allowed to adhere overnight. This cell density was selected based on preliminary assays that assessed cell growth rate and optimal absorbance readings in cell viability assays. Experimental setup consisted of exposing cells to increasing concentrations of propranolol and metoprolol (10, 25, 50, 100, 125, 150, 200 and 250 µM), cisplatin and flutamide (1, 5, 20, 50, 100, 150, 200 and 250 µM); atenolol and carvedilol (0.1, 1, 5, 10, 20, 50, 75 and 100 µM). The cell viability was assessed after 24, 48 and 72 h. Since pharmaceutical clearance rates in this model are not well defined, effects were assessed performing test media renewal every 24 h and without media renewal. Test controls were made with media with ultra-pure water or DMSO, depending on the tested drug. Viability was determined in at least three independent experiments, using three technical replicates.

2.3.2. Combined Exposure

Based on the results of individual exposures, propranolol (β-blocker) was selected to be tested in combined exposures with cisplatin and flutamide to assess the effects of binary mixtures on cellular viability of 22Rv1, PC3 and PNT-2. Cells were exposed for 48 h to different concentrations of the tested pharmaceuticals based on a factorial design. MTT viability assay was performed to determine the impact of these treatments on cell viability. As in the individual exposure tests, controls were made with media with ultra-pure water or DMSO, depending on the tested drug. The cytotoxicity was determined in at least three independent experiments, using three technical replicates.

2.4. Viability Assays

2.4.1. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

MTT (Sigma-Aldrich) was dissolved in PBS pH 7.36, to obtain a stock solution of 5 mg/mL, sterilized by filtration (0.22 µm pore PES filter), aliquoted and stored at -20 °C, protected from light. After each exposure period, test media was removed and cells carefully were washed with PBS, pH 7.36. MTT was further diluted (1:10) in PBS pH 7.36 and added to the plate well, with an incubation period of 2 h. After the incubation period, MTT solution was removed, and formazan crystals were solubilized by the addition of DMSO. The absorbance of the samples was measured using a microplate reader (Multiskan Spectrum - Thermo Scientific) at 570 nm, maximum absorbance, and 690 nm as a baseline. Viability was expressed as a percentage of respective control (Riss et al., 2016).

2.5. Data Analysis

Lethal doses (LD_{50}, LD_{25} and LD_{10}) were estimated using a nonlinear regression fitting curve, with variable slope (four parameters) using GraphPad 9 prism software. The effects of media renewal were tested with a paired t-test (significant differences were assumed for $p < 0.05$). In order to understand if there were significant differences ($p < 0.05$) between exposure time-points, a paired one-way ANOVA was performed after required assumptions tested, followed by a Tukey test. To understand if there were significant differences ($p < 0.05$) between cells responses to the pharmaceuticals at 24, 48 and 72 h, a paired one-way ANOVA was performed, followed by a Tukey test. The potential interaction of the pharmaceuticals in the combined exposure assays was analysed with MixTox model (Jonker et al., 2005).

3. Results

3.1. β-Blockers

3.1.1. β1-Blockers

The effects of the tested pharmaceuticals, atenolol and metoprolol, were tested in the four cell lines and the results are presented in Figure 1. In general, all cell lines presented a small reduction of viability in a time and concentration dependent manner. Since no relevant decrease in cell viability was observed, even at high concentrations, data did not show a good fit for the nonlinear regression model. LDs for both pharmaceuticals in all cell lines were not estimated. However, cell viability decreases between 15 and 40% were observed in cell exposed to atenolol and metoprolol. Viabilities were always higher at 24 h than at 48 and 72 h, for both pharmaceuticals. Overall, metoprolol was shown to present the highest statistical difference between all time-points with the exception for 22Rv1 cell line that was not affected (Supplementary Table 1). Paired one-way ANOVA analysis further confirms differences between metoprolol across time points, demonstrating a positive statistical significance in all cell lines with the exception of 22Rv1 (Supplementary Table 1). For atenolol, differences across all time-points were only statistically relevant for PNT-2 and LNCaP (Supplementary Table 1).

The assessment of the effect of media renewal on cell viability revealed that this is an important aspect to consider in the experimental design. In cell lines 22Rv1 and LNCaP, the medium renewal at 48 h and 72 h made a statistically significant effect on cell viability. In the case of cell lines PNT-2 and PC3 only the change of medium at 72 h made a statistically relevant difference, for both atenolol and metoprolol, with the change at 48 h being relevant only for metoprolol in PNT-2 (Supplementary Table 2 and 3). Both metoprolol and atenolol demonstrated a decrease in cellular viability at higher concentrations in the medium renewal. At the maximum concentration of atenolol (100 μM), 22Rv1 cell line demonstrated a reduction of viability of 10% at 48 h (48 h – 71.1% vs 48 h with change – 64.1%) and 2.5% at 72 h (72 h – 79.5% vs 72 h with change – 77.03%). For metoprolol, at the highest concentration (250 μM), the effects of media renewal were not so expressive at 48 h, with a reduction of 3.5% (48 h – 66.6% vs 48 h with change – 63.0%) and a 5% increase at 72 h (72 h – 59.4% vs 72 h with change – 64.1%). For LNCaP the result was similar with atenolol (at 100 μM) demonstrating an increase of 3% at 48 h (48 h – 74.7% vs 48 h with change 77.3 %) and 1% at 72 h (72 h – 78.4% vs 72 h with change – 79.3 %).

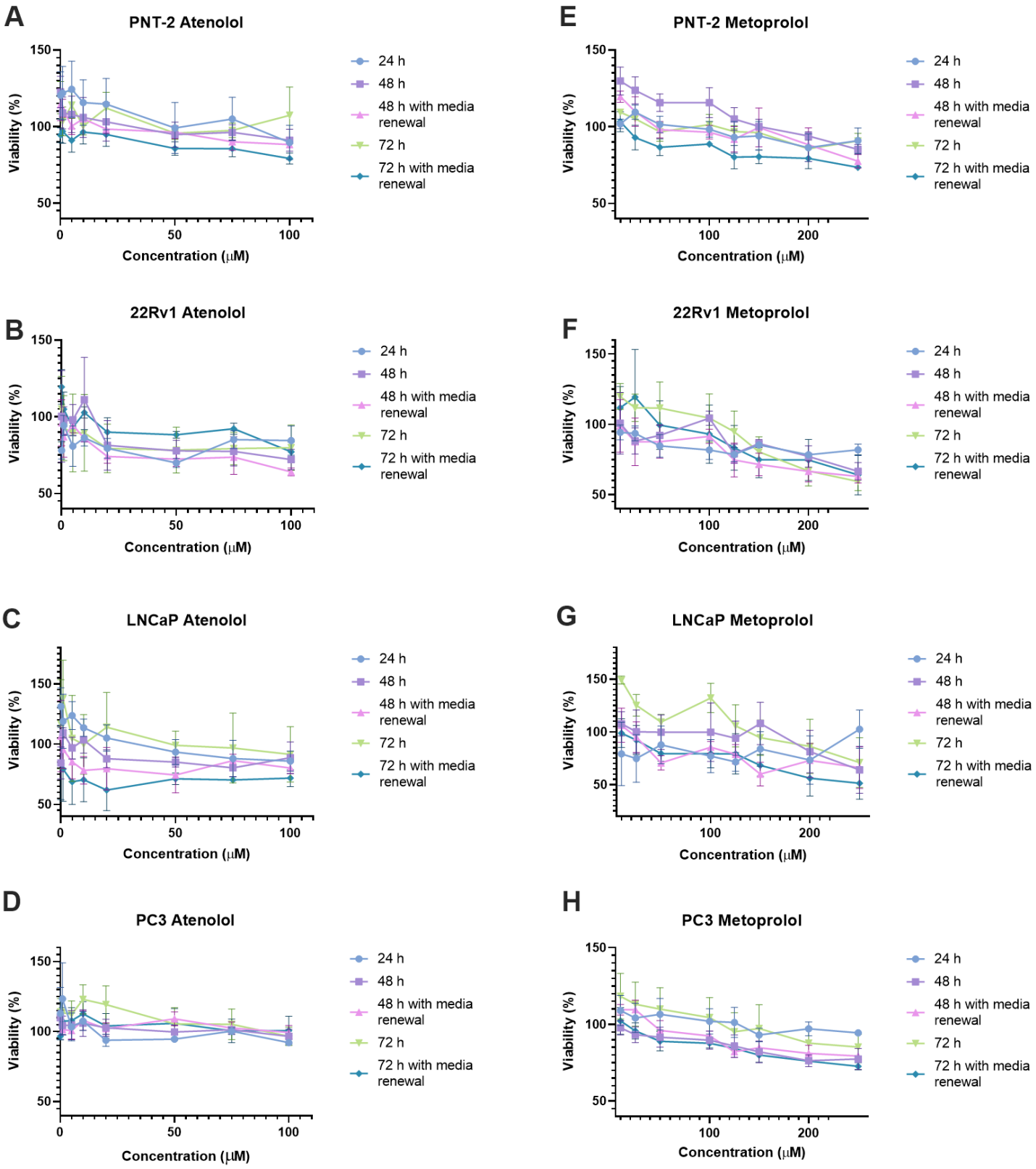

Figure 1: Viability of PNT-2, 22Rv1, LNCaP and PC3 cells exposed to atenolol (A-D) or metoprolol (E-H) for 24, 48 and 72 h. 48 and 72 h with media renewal correspond to the cells that were submitted to test media renewal every 24 h. Results are presented as mean of percentage of control ± standard error (n = 3 replicates).

3.1.2. Non-selective β-Blocker

The cytotoxic effect of propranolol and carvedilol on the tested cell lines is presented in Figure 2. Propranolol and carvedilol cytotoxicity increased in a time and concentration dependent manner. Unlike atenolol and metoprolol, the exerted cytotoxicity was extensive in all tested cell lines with propranolol and carvedilol with viability values close to 0 at the highest concentration (250 and 100 μM, respectively). By analysing the graphs of Figure 2, is possible to see that, in general, at 24 h the viability is always higher in comparison with the other time-points, in accordance with the results from atenolol and metoprolol. The estimated LD_{50}, LD_{25} and LD_{10} at different time-points for both pharmaceuticals are presented in Tables 5 and 6. The analysis of the LD values shows that 22Rv1 (propranolol: LD_{50} at 72 h – 54.639 μM; carvedilol: LD_{50} at 72 h – 14.990 μM) was the most sensitive cell line and PC3 (propranolol: LD_{50} at 72 h – 183.899 μM; carvedilol: LD_{50} at 72 h – 31.368 μM) was the most resistant to treatment in all the time-points tested for both propranolol and carvedilol. No significant differences in the effects of propranolol and carvedilol across time-points were observed in 22Rv1 and LNCaP but, PNT-2 and PC3, significant effects were found (Supplementary Tables 2 and 3). This result is further verified by analysis of the cytotoxic curves on Figure 3, where differences between the different time-point curves in PNT-2 (Figure 3A and 3E) and PC3 (Figure 3D and 3H) were noted.

When analysing the effect of renewing the media every 24 hours, contrary to what was found with atenolol and metoprolol, changes of media at 48 h time point did not exert statistically significant differences in all the cell lines tested, to both pharmaceuticals (Supplementary Table 2). At time-point 72 h, media renewal of propranolol was significantly different in 22Rv1and PC3, while for carvedilol in LNCaP and PC3 (Supplementary Table 3).

When the analysis is focused in one specific time-point (Figure 4) is easy to correlate the calculated LDs (Table 5 and 6) with the paired one-way ANOVA results (Supplementary Table 4). According to Table 5, the values for the LD_{50} of carvedilol at 24 h are similar between cell lines with the exception of 22Rv1 (LD_{50} of 25.701μM) that showed a higher sensitivity. For propranolol, in all time-points analysed, there were statistically relevant differences between cell lines (Supplementary Table 1), which is visible in Figure 3 (E to H) with the cytotoxic curves assuming several different shapes.

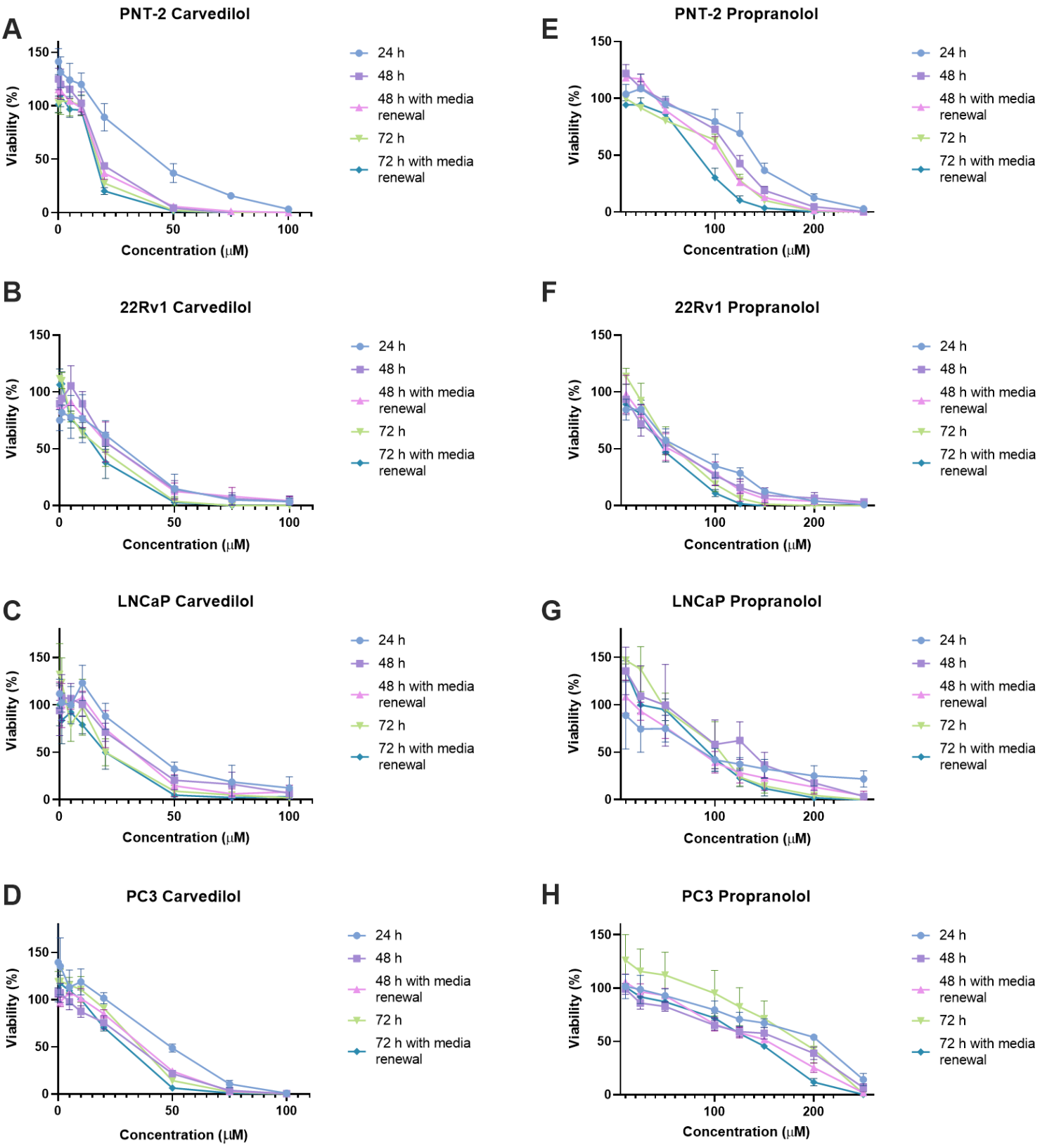

Figure 2: Viability of PNT-2, 22Rv1, LNCaP and PC3 cells exposed to carvedilol (A-D) or propranolol (E-H) for 24, 48 and 72 h. 48 and 72 h with media renewal correspond to the cells that were submitted to test media renewal every 24 h. Results are presented as mean of percentage of control ± standard error (n = 3 replicates).

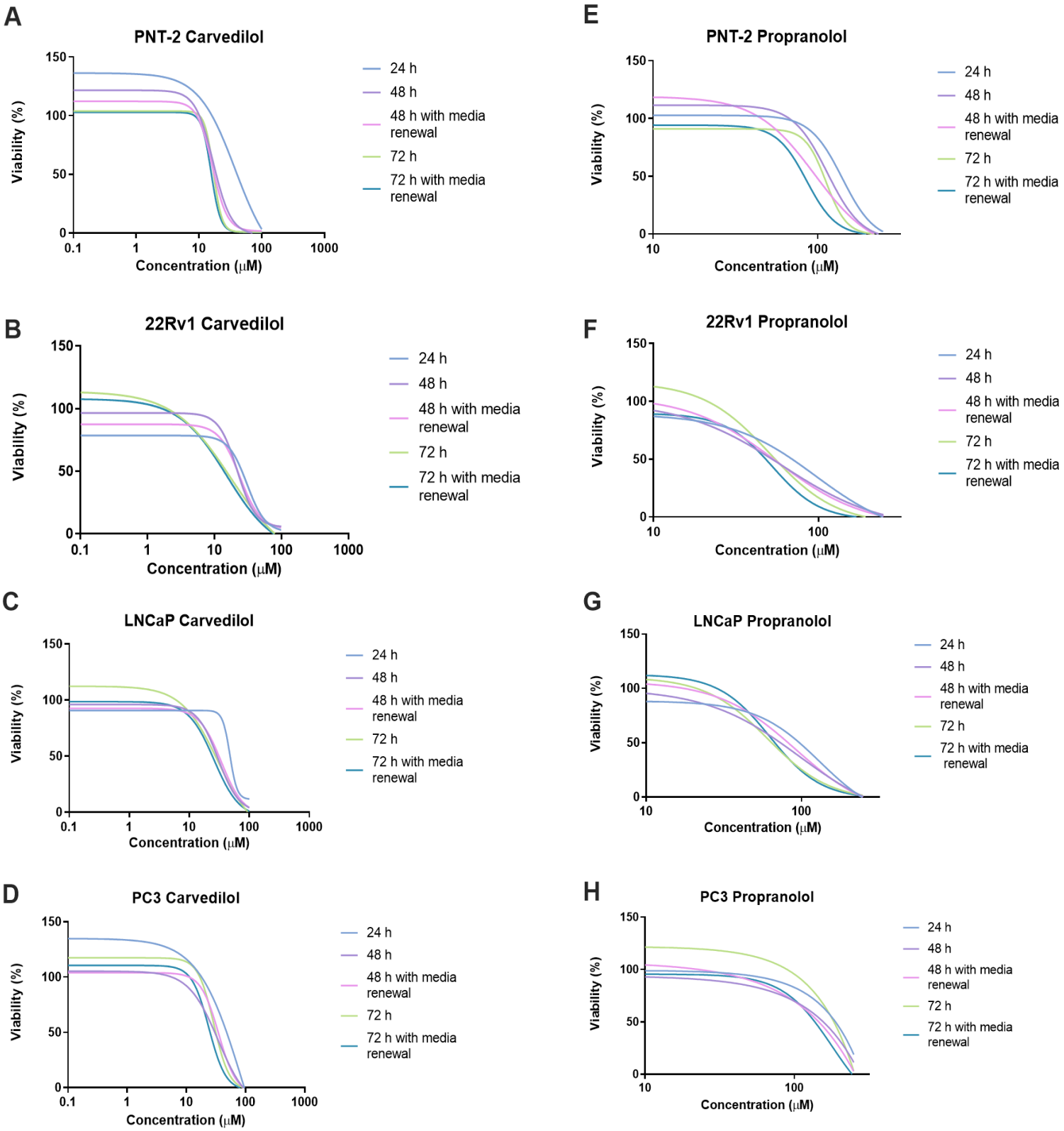

Figure 3: Dose-response nonlinear regression curve (four-parameter logistic curve) of PNT-2, 22Rv1, LNCaP and PC3 cells exposed to carvedilol (A-D) or propranolol (E-H) for 24, 48 and 72 h. 48 and 72 h with media renewal represent the plates where there was a 24h renewal of test media. Results are presented as a percentage of control.

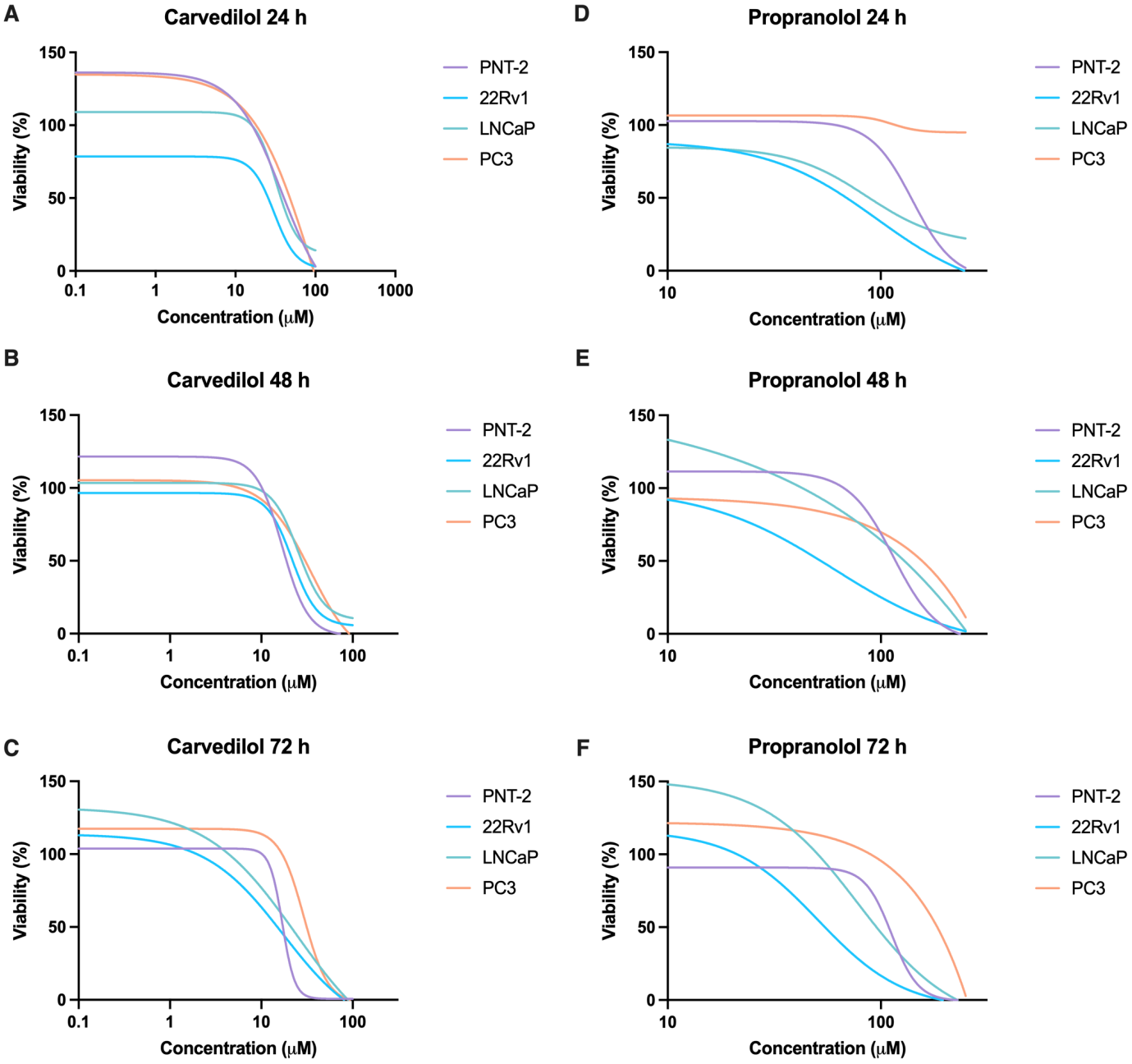

Figure 4: Dose-response nonlinear regression curve (four-parameter logistic curve) of PNT-2, 22Rv1, LNCaP and PC3 cells exposed to carvedilol (A-D) or propranolol (E-H) for 24, 48 and 72 h. Results are presented as a percentage of control.

Table 5: Estimated lethal doses (LD_{50}, LD_{25} and LD_{10}) of carvedilol for PNT-2, 22Rv1, LNCaP and PC3 cells after 24, 48 and 72 h exposure to carvedilol. 48 and 72 h with media renewal represent the plates where their test media was renewed every 24h. LDs were calculated through interpolation of data in a nonlinear regression (four-parameter logistic dose-response curve). Values missing indicate that LDs were out of the curve range.

Carvedilol	μM	24 h	48 h	48 h with media renewal	72 h	72 h with media renewal
PNT-2	LD_{50}	40.480	18.818	17.541	17.211	15.804
	LD_{25}	26.621	14.354	13.592	14.630	13.192
	LD_{10}	20.076	11.914	11.209	12.665	11.186
22Rv1	LD_{50}	25.701	21.994	22.128	14.990	13.836
	LD_{25}	11.588	15.021	12.816	6.872	6.628
	LD_{10}	------	9.719	------	3.682	3.525
LNCaP	LD_{50}	47.867	29.104	31.262	27.328	24.305
	LD_{25}	39.151	18.059	18.276	15.471	14.210
	LD_{10}	25.115	10.167	7.644	9.762	7.983
PC3	LD_{50}	47.980	30.241	34.589	31.368	25.160
	LD_{25}	31.748	17.855	24.511	24.159	18.571
	LD_{10}	23.325	11.029	17.925	20.019	14.623

Table 6: Estimated lethal doses (LD_{50}, LD_{25} and LD_{10}) of propranolol for PNT-2, 22Rv1, LNCaP and PC3 cells after 24, 48 and 72 h exposure to propranolol. 48 and 72 h with media renewal represent the plates where their test media was renewed every 24h. LDs were calculated through interpolation of data in a nonlinear regression (four-parameter logistic dose-response curve). Values missing indicate that LDs were out of the curve range.

Propranolol	μM	24 h	48 h	48 h with media renewal	72 h	72 h with media renewal
PNT-2	LD_{50}	139.001	117.212	97.630	108.953	82.806
	LD_{25}	110.623	94.561	71.058	89.799	64.104
	LD_{10}	89.282	79.910	56.195	58.018	44.897
22Rv1	LD_{50}	69.208	52.333	53.391	54.639	47.895
	LD_{25}	31.530	25.367	29.464	36.942	30.117
	LD_{10}	------	11.950	17.237	27.681	------
LNCaP	LD_{50}	97.157	73.326	82.191	64.366	67.427
	LD_{25}	51.821	37.296	49.186	42.154	47.905
	LD_{10}	------	17.950	31.197	30.168	36.963
PC3	LD_{50}	188.569	156.204	145.672	183.899	137.149
	LD_{25}	125.484	83.528	87.254	141.890	92.730
	LD_{10}	72.012	26.066	49.899	112.348	54.149

3.2. Cytostatic Drugs

3.2.1. Cisplatin

The cytotoxic effect of cisplatin on the tested cell lines is presented in Figure 5. Cisplatin cytotoxicity increased in a time-dependent manner as observed for the other tested drugs. 22Rv1 presented a range of reduction of viability from 20% at 24 h to 50% at 48 h and 70% at 72 h (Figure 5), demonstrating that this cell line is, as observed for carvedilol and propranolol, the most sensitive line to cisplatin at 72 h. PC3 presented a reduction of viability of 32% at 24 h but at 48 and 72 h this reduction was of 46% and 57% (Figure 5), respectively, demonstrating to be the most resistant cell line to cisplatin

cytotoxicity. The estimated LD_{50}, LD_{25} and LD_{10} at different time-points are presented in Table 7. These values follow in accordance with what is visible in the graphs of Figure 6, with 72 h LD_{50} of 22Rv1 being the smaller and LD_{50} of PC3 being the highest. In some cell lines, especially in 22Rv1, the model did not estimate LDs at 24 h (Table 7). When considering each time point (Figure 7; Supplementary Table 4), it is clear to Cisplatin at 48 and 72 h that the cytotoxic curves diverge, demonstrating statistical differences, specially between 22Rv1 and PC3, at 72 h.

Media renewal every 24 h showed to be statistically relevant for 22RV1 and LNCaP in both time-points and in PC3 only at 72 h (Supplementary Table 2 and 3).

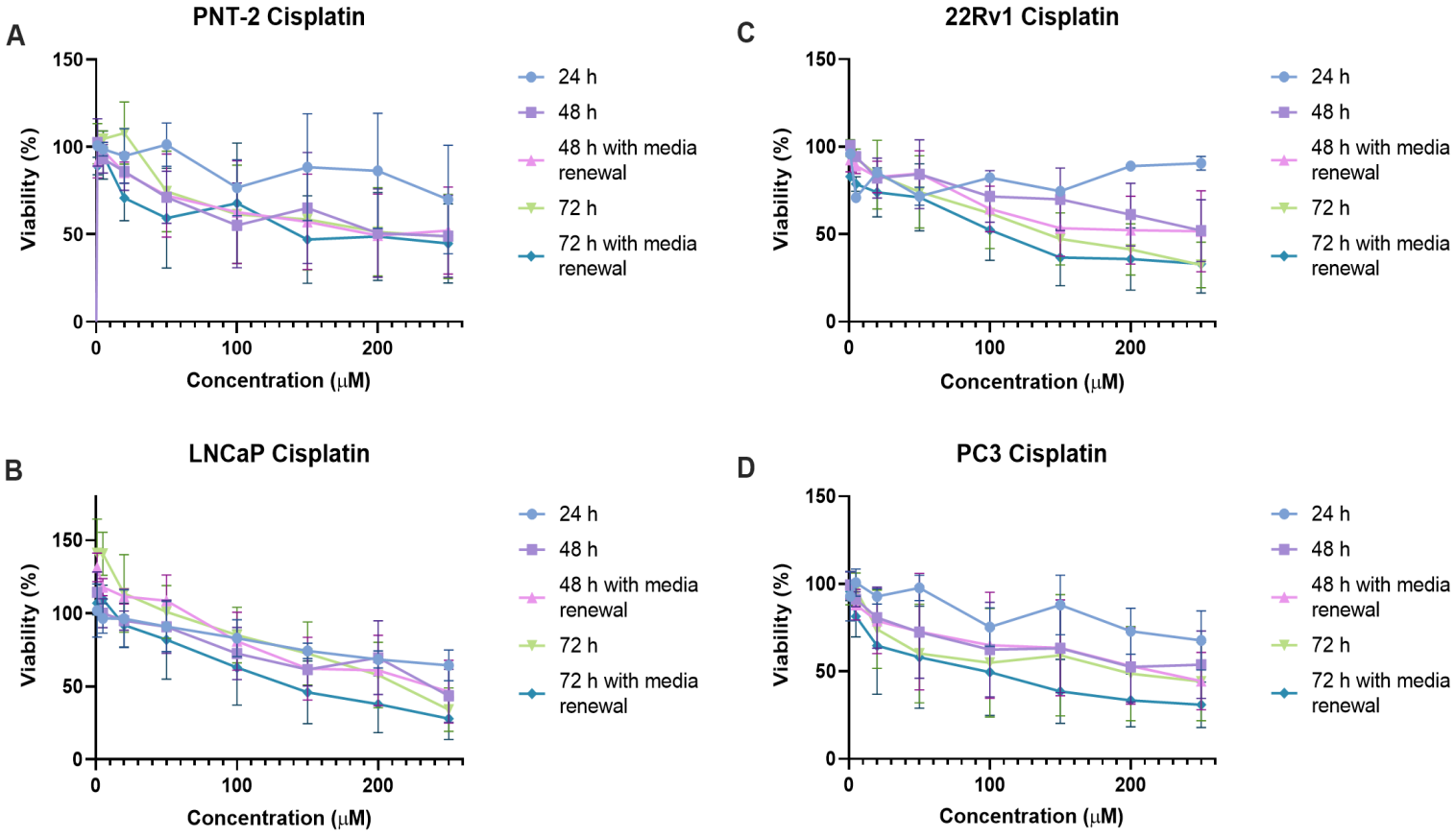

Figure 5: Viability of PNT-2, 22Rv1, LNCaP and PC3 cells exposed to cisplatin (A-D) for 24, 48 and 72 h. 48 and 72 h with media renewal correspond to the cells that were submitted to test media renewal every 24 h. Results are presented as mean of percentage of control ± standard error (n = 3 replicates).

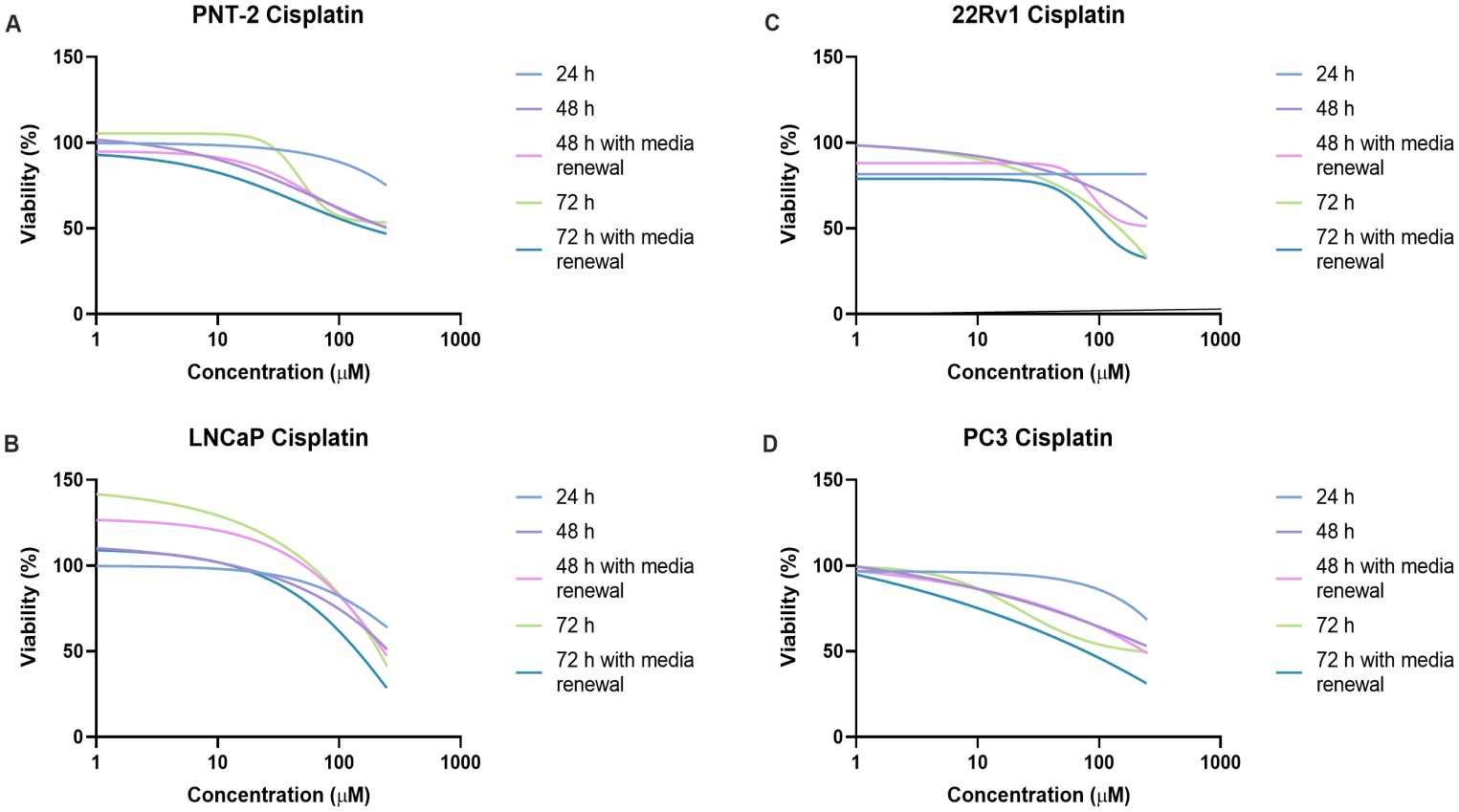

Figure 6: Dose-response nonlinear regression curve (four-parameter logistic curve) of PNT-2, 22Rv1, LNCaP and PC3 cells exposed to cispaltin (A-D) for 24, 48 and 72 h. 48 and 72 h with media renewal represent the plates where there was test media renewal every 24 h. Results are presented as mean of percentage of control.

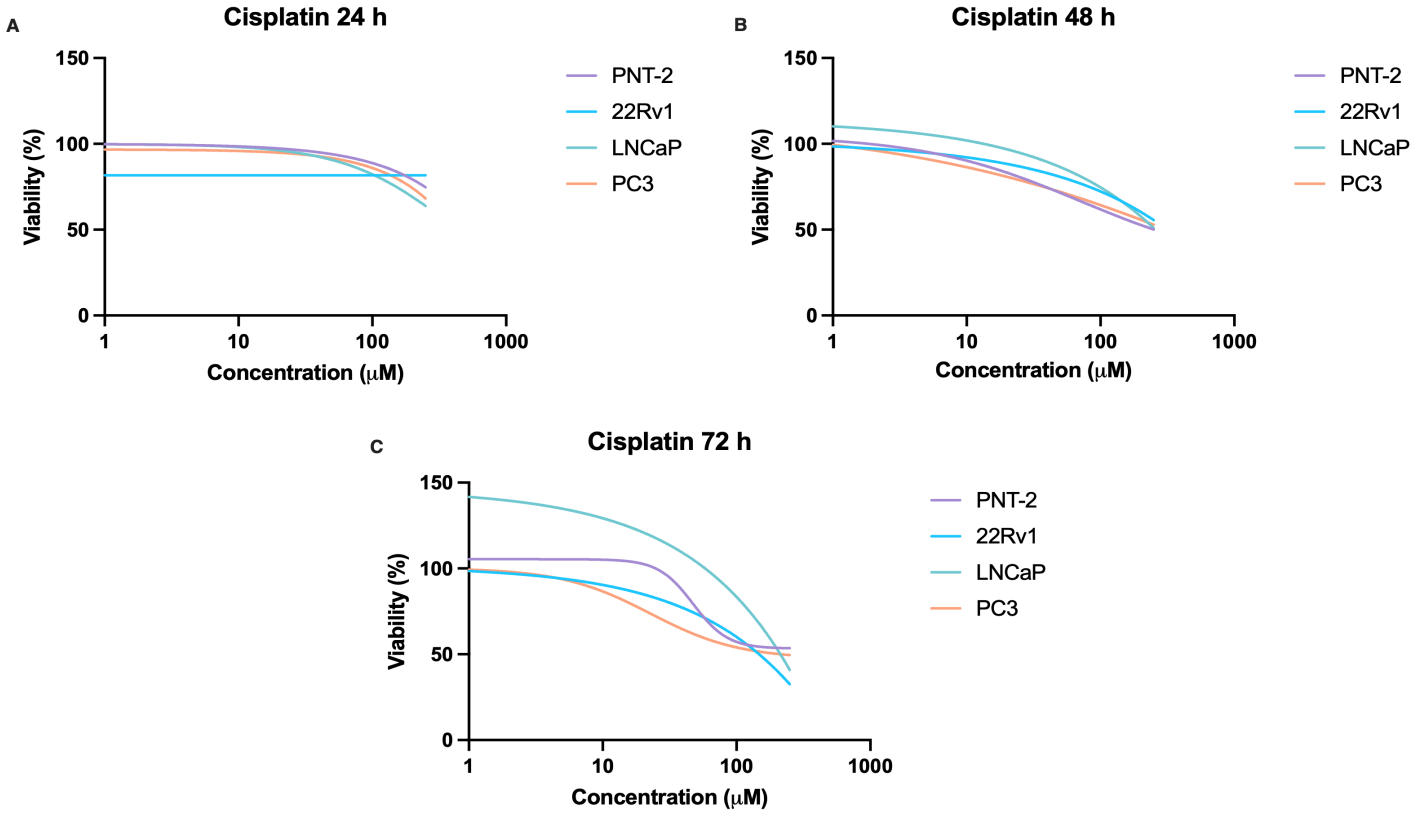

Figure 7: Dose-response nonlinear regression curve (four-parameter logistic curve) of PNT-2, 22Rv1, LNCaP and PC3 cells exposed to cisplatin for 24 (A), 48 (B) and 72 (C) h. Results are presented as a percentage of control.

Table 7: Estimated lethal doses (LD_{50}, LD_{25} and LD_{10}) of cisplatin for PNT-2, 22Rv1, LNCaP and PC3 cells after 24, 48 and 72 h exposure to cisplatin. 48 and 72 h with media renewal represent the plates where their test media was renewed every 24h. LDs were calculated through interpolation of data in a nonlinear regression (four-parameter logistic dose-response curve). Values missing indicate that LDs were out of the curve range.

Cisplatin	μM	24 h	48 h	48 h with media renewal	72 h	72 h with media renewal
PNT-2	LD_{50}	536.222	252.210	281.539	------	172.507
	LD_{25}	246.595	38.608	44.923	52.408	21.259
	LD_{10}	88.232	10.236	12.723	36.560	2.990
22Rv1	LD_{50}	------	314.395	------	147.568	102.993
	LD_{25}	------	82.142	74.782	45.626	37.431
	LD_{10}	------	15.160	------	10.661	------
LNCaP	LD_{50}	435.187	256.826	233.056	212.775	141.683
	LD_{25}	150.811	98.296	125.031	124.942	62.923
	LD_{10}	53.601	38.340	77.811	82.291	30.158
PC3	LD_{50}	403.360	310.073	234.706	214.681	77.181
	LD_{25}	192.946	37.277	40.021	21.092	10.201
	LD_{10}	64.896	5.984	5.446	7.547	1.920

3.2.2. Flutamide

The effects of flutamide on the viability of the tested cell lines are presented in Figure 8. Flutamide cytotoxicity increased in a time-dependent manner, with viabilities reducing up to 40% in all cell lines at 24 and 48 h and further to 60% at 72 h. The estimated LD_{50}, LD_{25} and LD_{10} at different time-points for both pharmaceuticals are presented in Tables 8. The model used to estimate the LDs values was not able to estimate them at 24 h. Therefore, it was not possible to ascertain a sensitive versus resistant cell line to the treatment.

Statistical analysis demonstrates that only LNCaP cell line did not exhibit differences across time points, with relevance for the other cell lines that show statistical differences between 48 h and 72 h (Figure 9 and Supplementary Table 1).

Media renewal only impacted the 48 h time point in PNT-2 and 22Rv1 (Supplementary Table 2). A comparison of the different cell lines in each time point reveals that flutamide exerts the major effect at 24 h, cell lines exhibiting a different response and PC3 being statistically different from 22Rv1 and PNT-2. However, this tendency is not observed at longer exposure periods (Figure 10 and Supplementary Table 4) showing a convergent effect across cell lines.

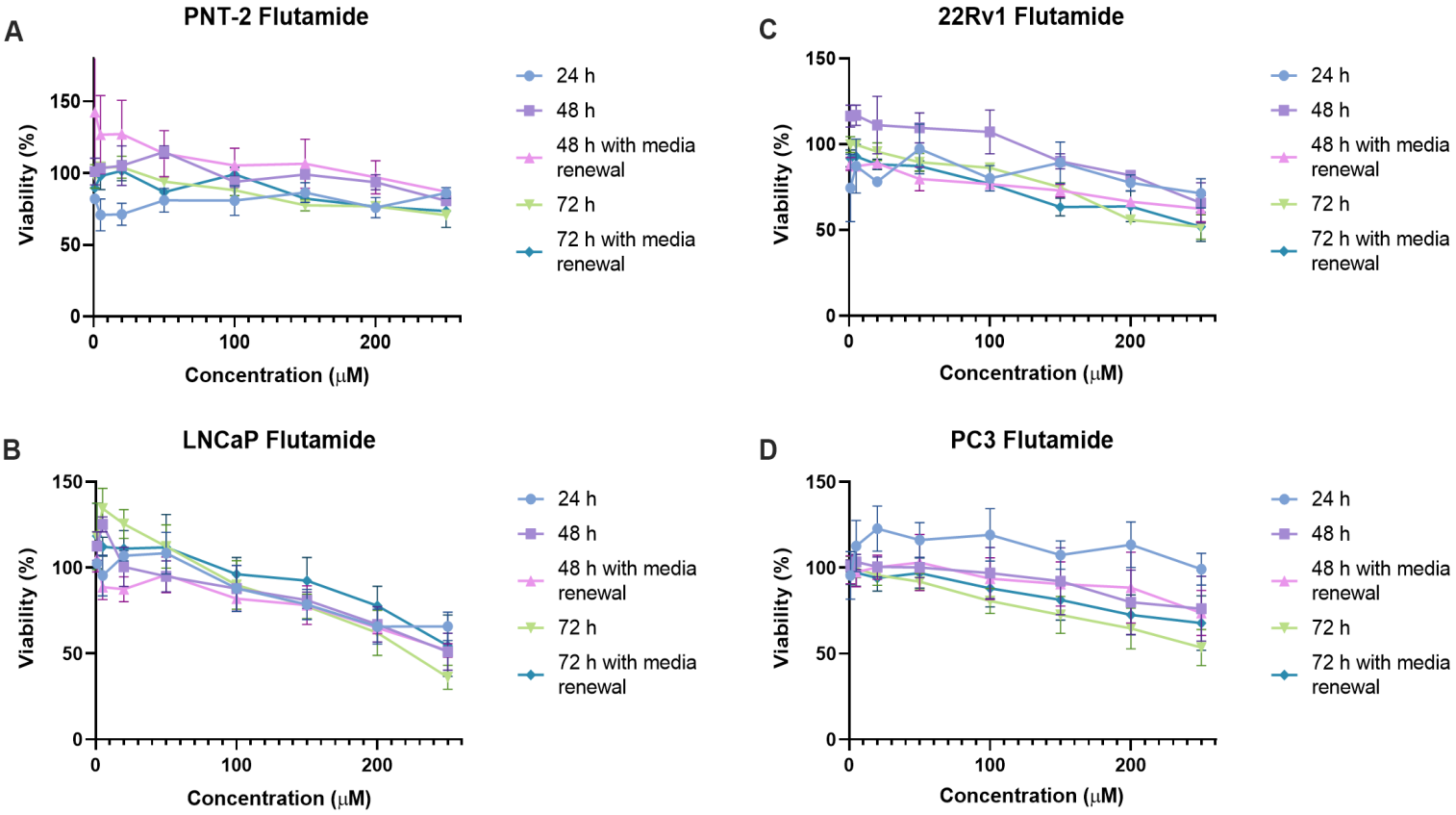

Figure 8: Viability of PNT-2, 22Rv1, LNCaP and PC3 cells exposed to flutamide (A-D) for 24, 48 and 72 h. 48 and 72 h with media renewal correspond to the cells that were submitted to test media renewal every 24 h. Results are presented as mean of percentage of control ± standard error (n = 3 replicates).

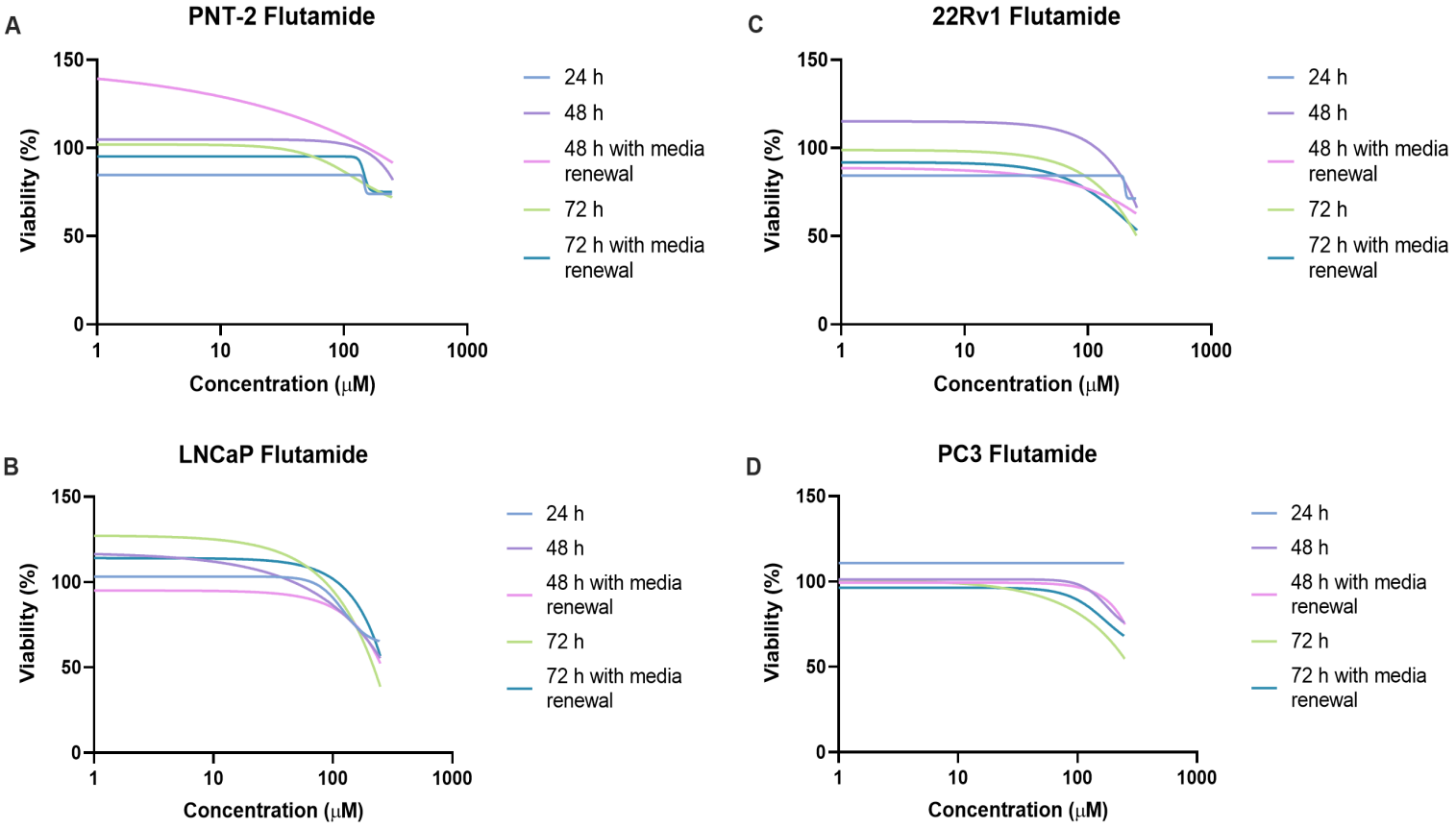

Figure 9: Dose-response nonlinear regression curve (four-parameter logistic curve) of PNT-2, 22Rv1, LNCaP and PC3 cells exposed to flutamide (A-D) for 24, 48 and 72 h. 48 and 72 h with media renewal represent the plates where there was test media renewal every 24 h. Results are presented as mean of percentage of control.

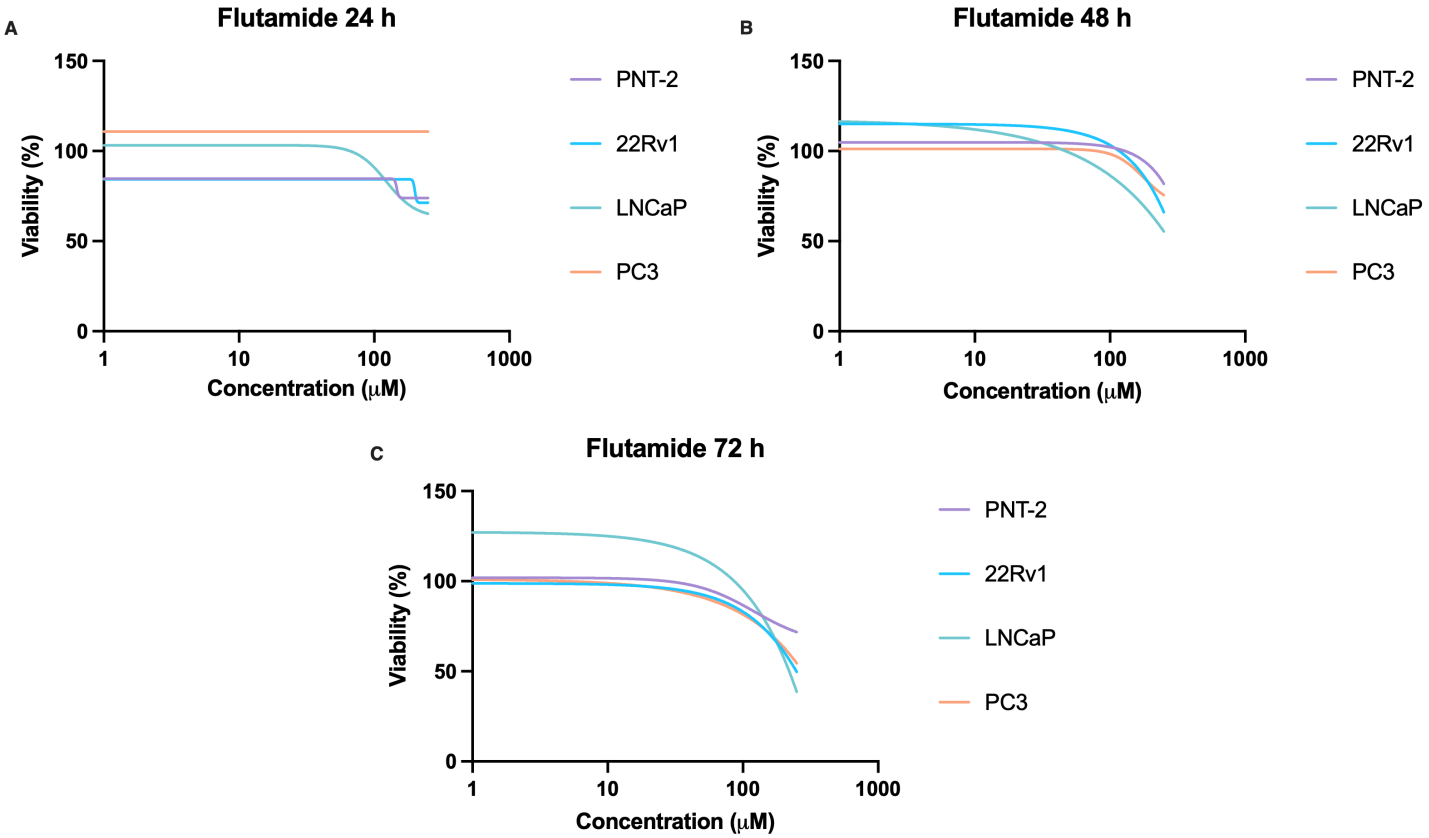

Figure 10: Dose-response nonlinear regression curve (four-parameter logistic curve) of PNT-2, 22Rv1, LNCaP and PC3 cells exposed to flutamide for 24 (A), 48 (B) and 72 (C) h. Results are presented as a percentage of control.

Table 8: Estimated lethal doses (LD_{50}, LD_{25} and LD_{10}) of flutamide for PNT-2, 22Rv1, LNCaP and PC3 cells after 24, 48 and 72 h exposure to flutamide. 48 and 72 h with media renewal represent the plates where their test media was renewed every 24h. LDs were calculated through interpolation of data in a nonlinear regression (four-parameter logistic dose-response curve). Values missing indicate that LDs were out of the curve range.

Flutamide	μM	24 h	48 h	48 h with media renewal	72 h	72 h with media renewal
PNT-2	LD_{50}	------	361.889	1240.377	------	------
	LD_{25}	152.388	279.343	527.581	195.949	------
	LD_{10}	------	207.571	270.304	82.129	139.107
22Rv1	LD_{50}	------	300.982	405.945	248.645	283.721
	LD_{25}	202.430	219.424	116.561	137.727	105.665
	LD_{10}	------	162.223	------	64.558	22.292
LNCaP	LD_{50}	------	278.930	258.382	220.067	266.702
	LD_{25}	152.318	151.495	154.709	154.065	199.087
	LD_{10}	102.532	85.261	64.863	113.839	149.563
PC3	LD_{50}	------	------	332.414	275.430	------
	LD_{25}	------	257.381	249.246	135.695	185.045
	LD_{10}	------	152.497	168.725	55.434	95.046

3.3. Mixtures

3.3.1. Binary Mixture

Based on the data obtained for individual exposures, the viability of cell lines PNT-2, 22Rv1 and PC3 was tested when submitted to binary mixtures of drugs. Thus, binary mixtures of propranolol, cisplatin and flutamide were tested on selected prostate cell lines. The data analysis using the ModelTox allowed the assessment of potential independent action (IA), synergism or antagonism (S/A), dose ratio-dependent deviation (DR) or dose level-dependent deviation (DL). The data of the cell viability after the model is presented in Figures 11, 12 and 13.

In PNT-2, the data from mixture of cisplatin and propranolol (Figure 11A) the DR model was the most fitting, with parameters presented in Table 9. These values support an interaction involving antagonism at lower concentrations of propranolol and synergism at higher concentrations. In the mixture of flutamide and propranolol (Figure 11B) S/A model was the most fitting, with obtained parameter presented in Table 9, and supporting an antagonistic effect. The DL model was validated for the mixture of flutamide, and cisplatin (Figure 11C) and the obtained parameters are presented in Table 9. The model supports an antagonistic effect between these pharmaceuticals.

In 22Rv1, the mixture of cisplatin and propranolol (Figure 12A) also fitted better in the DL model, with parameters (Table 10), supporting a synergism at lower concentrations of propranolol and antagonism at higher concentrations. For the mixture of flutamide and propranolol (Figure 12B), the S/A model an antagonistic effect whereas for the mixture of flutamide and cisplatin (Figure 12C), the IA model show independent effects (additive).

In PC3, all mixtures (Figure 13) used the IA model (Table 11), which means that the combinations of pharmaceuticals have independent effects (additive).

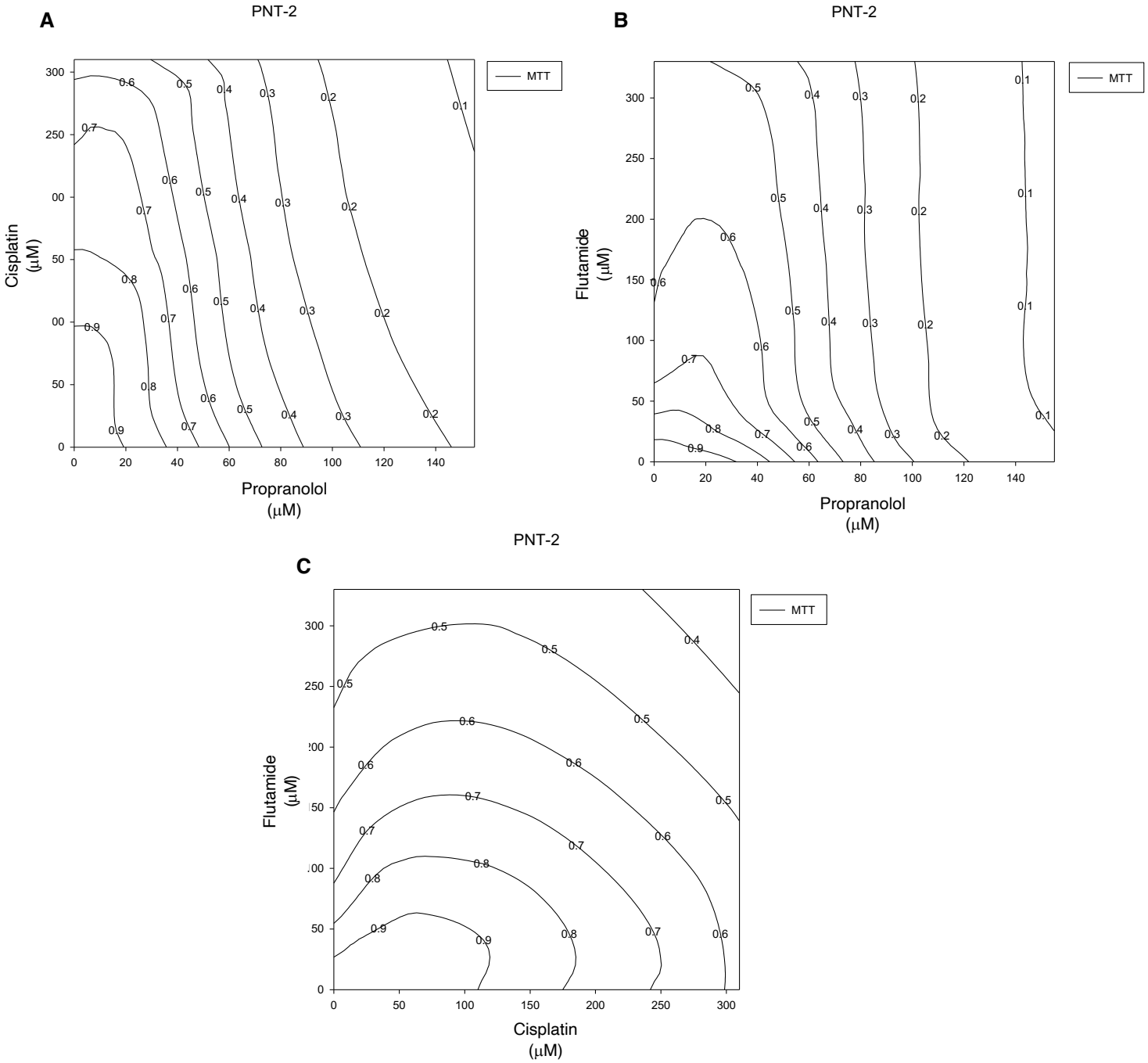

Figure 11: Dose-response after 48 h binary mixture propranolol and cisplatin (A), propranolol and flutamide (B) or cisplatin and flutamide (C) in cells PNT-2.

Table 9: Parameters obtained for each model in each binary mixture for PNT-2. DR: dose ratio-dependent deviation; S/A: synergism or antagonism; DL: dose level-dependent deviation.

	Model	a	b
Cisplatin + Propranolol	DR	2.149	-3.830
Flutamide + Propranolol	S/A	0.743	-------
Flutamide + Cisplatin	DL	3.748	0.911

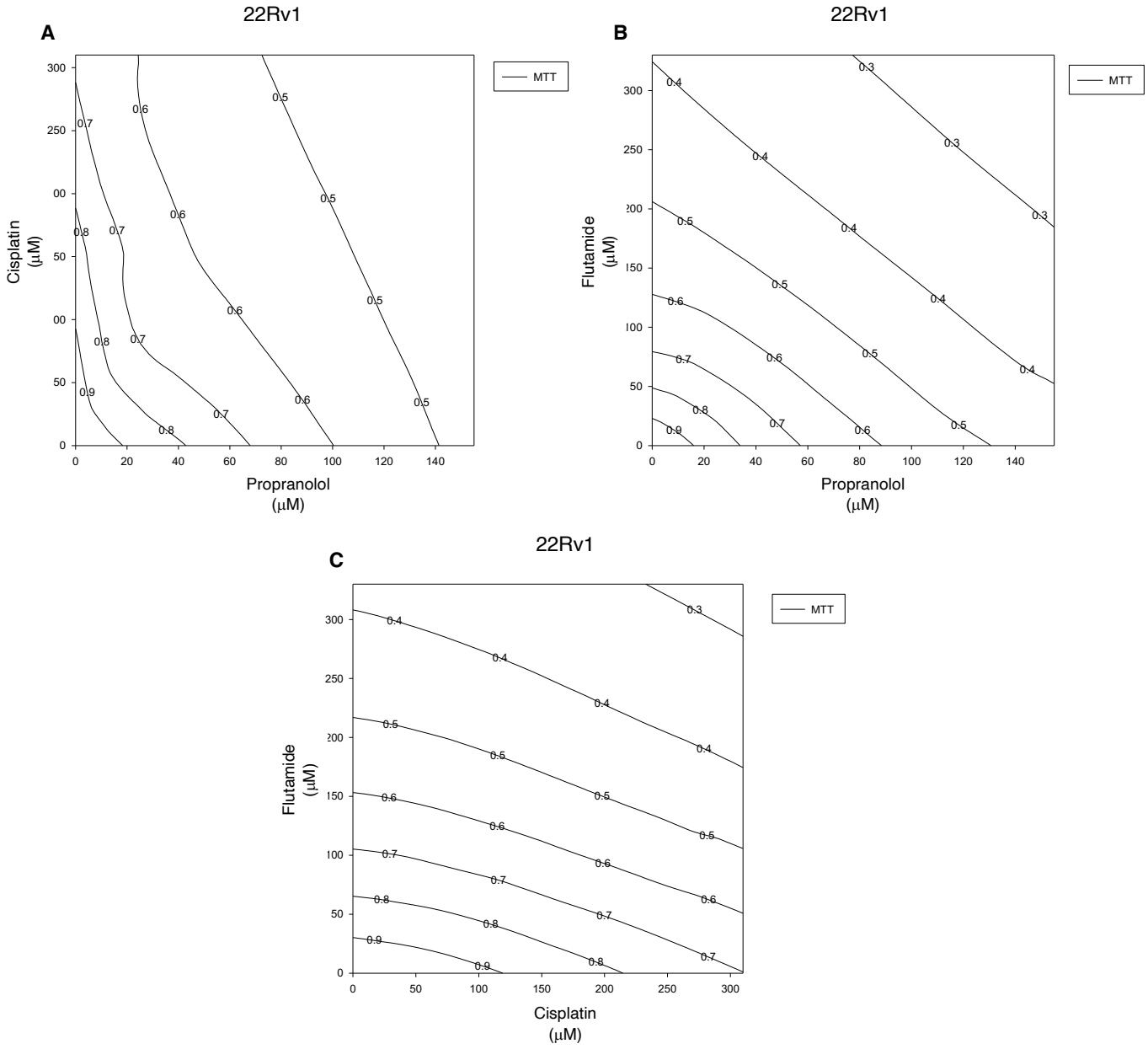

Figure 12: Dose-response after 48 h binary mixture propranolol and cisplatin (A), propranolol and flutamide (B) or cisplatin and flutamide (C) in cells 22Rv1.

Table 10: Parameters obtained for each model in each binary mixture for 22Rv1. DL: dose level-dependent deviation; DR: dose ratio-dependent deviation; IA: independent action.

	Model	a	b
Cisplatin + Propranolol	DL	-2.278	2.100
Flutamide + Propranolol	S/A	0.700	-------
Flutamide + Cisplatin	IA	-------	-------

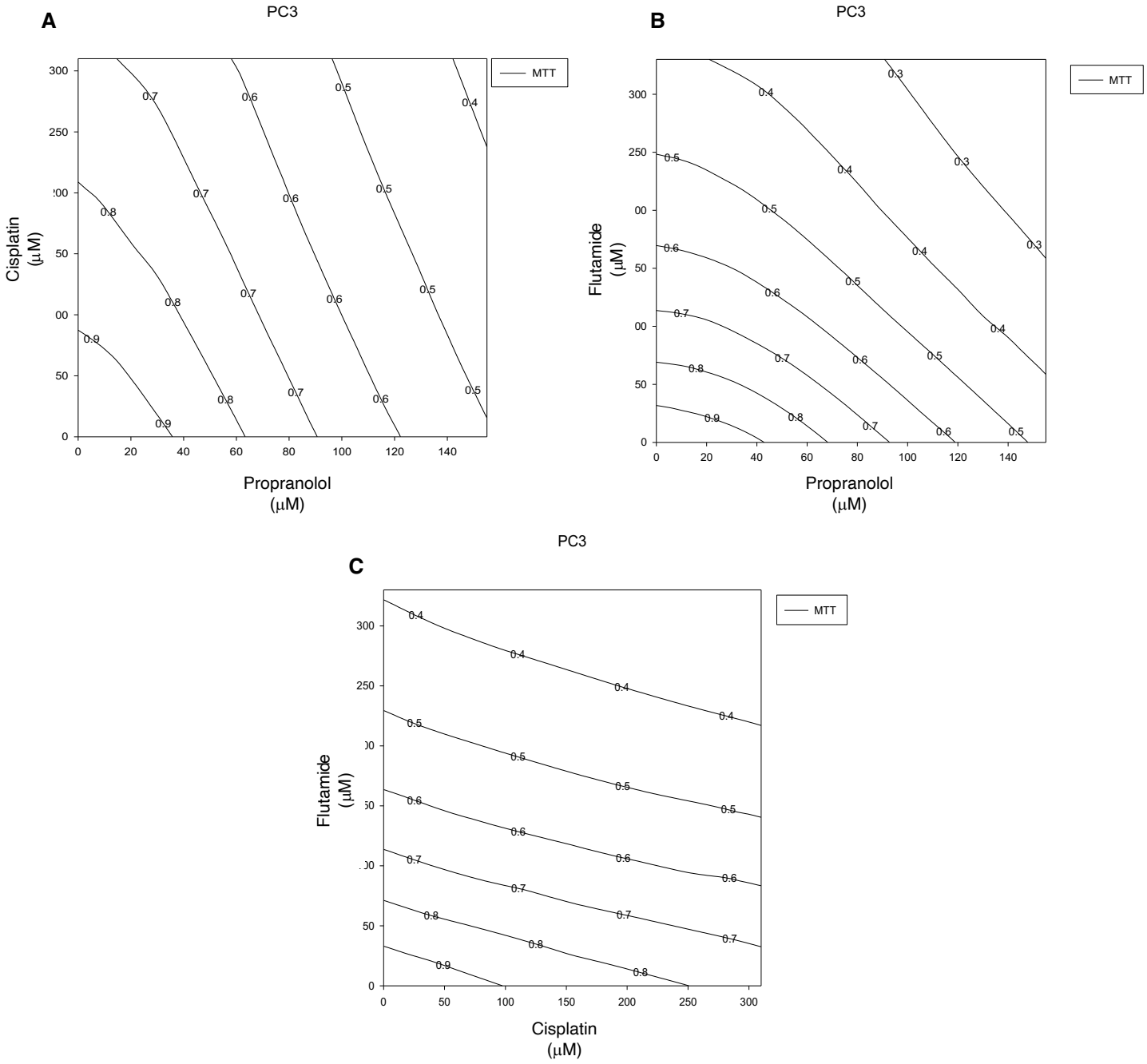

Figure 13: Dose-response after 48 h binary mixture propranolol and cisplatin (A), propranolol and flutamide (B) or cisplatin and flutamide (C) in cells PC3.

Table 11: Parameters obtained for each model in each binary mixture for PC3. IA: independent action.

	Model	a	b
Cisplatin + Propranolol	IA	-------	-------
Flutamide + Propranolol	IA	-------	-------
Flutamide + Cisplatin	IA	-------	-------

4. Discussion

In vitro methodologies have been increasingly used in biomedical research. Cell lines, in particular, have been used for many years to study diverse biological aspects in cancer (e.g., molecular pathways of signalling and new treatments). Considering the study goals, different approaches have been considered. For example, it may be important to have cell lines representative of different stages of cancer development as their characteristics may be different, so are the responses to the treatments to which they are submitted. Similarly, there is also the need to consider the effects on normal cell lines, as the goal of the treatment should be to induce maximum effects on cancer cells, without significantly compromising a normal cell line of the tissue of origin. This was the approach selected for this study. Thus, cell lines selected for this study were 22Rv1, which is representative of the first stage (solid tumour) of PCa, while LNCaP and PC3 represent metastatic cancer. However, LNCaP like 22Rv1 is sensitive to androgens, while PC3 cell line that represents the last stage of cancer where few treatments are available, does not express AR nor PSA and is androgen independent. Thus, significant differences in response to β-blockers could be expected. PNT-2 was selected as control cell line since it is a normal cell line for prostate and a potential treatment should have a minor impact on normal cells while being effective in compromising cancer cell propagation.

The hypothesis of my dissertation was based on the fact that β-adrenergic signalling has been found to regulate multiple cellular processes that contribute to the initiation and progression of cancer, including inflammation, angiogenesis, apoptosis, metastases, DNA damage repair, cellular immune response, and epithelial–mesenchymal transition (Cole & Sood, 2012; Zahalka et al., 2017). In order to obtain more relevant/robust data, the exposure conditions were also assessed as the build-up of metabolic products, degradation of the tested substance could influence the effects of the tested substances.

Furthermore, there was also the possibility that β-blockers alone could not have any effect on the cancer cell lines but could have a significant interaction with cytostatic drugs. In this sense, the effects of media renewal on the obtained data, effects of β-blockers individually, and effects of combined exposures were tested.

The results obtained in this study revealed that β1-blockers have limited cytotoxicity, assessed as decreased cell viability on all cell lines. This low toxicity may be associated with low levels of expression of the β1-adrenergic receptor in this cancer and over expression of β2-adrenergic receptor (Braadland et al., 2015; Nagmani et al., 2003). The activation of the β2-adrenergic receptor has been shown to promote prostate cancer cell progression in LNCaP, PC3 (Zhang et al., 2011), and DU145 cells (Barbieri et al., 2015). Even though β1-blockers have a low cytotoxicity to cancer cells at tested concentrations, they may have a better protective effect on other cells (e.g., cardiomyocytes), and thus could play an important role in minimizing the side effects of several pharmaceuticals used in cancer treatment. The lack of effects observed on the present study could also be associated with the levels tested. Further studies could explore the effects of higher concentrations, if within clinical safety levels.

Individual exposure of the different β-blockers (atenolol, carvedilol, metoprolol, and propranolol) showed different sensibilities between the cell lines. 22Rv1 was the most sensitive cell line whereas PC3 was the most resistant. Overall propranolol and carvedilol were the most toxic β-blockers. In terms of the cytostatic drugs, cisplatin was, as expected, the most toxic to the tested cell lines, supporting its use as chemotherapeutic drug. Considering the sensitivity to cisplatin the tested cell lines can be ranked as 22Rv1 > LNCaP > PNT-2 > PC3. This higher resistance of PC3 supports the current knowledge that cisplatin is used in solid tumours, presenting decreased efficiency in metastatic cancers (Matos et al., 2012). However, cisplatin has been considered to treat metastatic castration-resistant prostate cancer in combination with other compounds (e.g., taxanes) (Budman et al., 2002; Matos et al., 2012). Gumulec et al. (2014) and Raudenska et al. (2019) that studied the effects of cisplatin on 22Rv1, PNT1A (another normal prostate cell line) and PC3, also found that 22Rv1 was the most sensitive cell line and PC3 the most resistant. PC3 resistance to flutamide is expected since this cell line does not express androgen receptor, primary known target of this pharmaceutical.

In vitro studies have shown similar effects as this study in other cancers. Carvedilol has been reported to prevent invasion in breast cancer cell lines. In the same study, a retrospective analysis of women that used carvedilol at breast cancer diagnosis

was performed, revealing that breast cancer-specific mortality was reduced (Gillis et al., 2021). Carvedilol has also shown cytotoxic effects on colorectal (Coelho et al., 2015) and neuroblastoma (Pasquier et al., 2013) cancer cells. Propranolol has shown cytotoxic effects in breast (Montoya et al., 2019; Szewczyk et al., 2012; Xie et al., 2019), melanoma (Kuang et al., 2018), colorectal (Coelho et al., 2015; Işeri et al., 2014), gastric (Koh et al., 2021) and hepatocellular (Işeri et al., 2014) cancer cells. Metoprolol and atenolol showed low anti-proliferative properties in neuroblastoma cells (Pasquier et al., 2013).

The study of the effects of media renewal revealed differences in all cell lines, at 48 and 72 h, for the different pharmaceuticals tested. This effect might be explained due to higher doses of the pharmaceutical available. There is also a combination of effects of the parental compound and its possible metabolites. In the absence of media renewal, the cells might be exposed to lower concentrations of the compound, as the time passes, since these pharmaceuticals may degrade easily, due to, for example, light exposure or their volatility. Other factors may also be responsible for the differences in cellular viability, such as, loss of cells during manipulation. Despite the observed higher effects of the tested substances under a media renewal design, for the combined exposure, the experimental design did not use this approach as it is not often used in other studies and a potential influence of manipulation on cell loss could influence the potential interactions.

Having in consideration the results from individual exposure, 22Rv1, PNT-2 and PC3 cells were selected for combined exposures. The compounds selected for these exposures were propranolol, since it is a pharmaceutical widely used and studied, cisplatin, since it is an antineoplastic drug used for the treatment of several cancers, including prostate, and flutamide, an androgen receptor blocker used in hormone therapy for the treatment of this cancer. Overall, the concentrations tested showed antagonism at lower concentrations of propranolol in PNT-2, while at the same concentrations of propranolol synergism was found in 22Rv1. These results suggest that propranolol can have a positive impact in treatment of this cancer, by protecting the normal cells and helping to prevent cancer cell propagation, promoting higher mortality, within certain levels. The obtained data show that the positive effects in the combined exposures are limited to low concentrations, because at high concentration it can have the opposite effect. The potential positive role of propranolol was also proposed in other *in vitro* studies, but mostly in individual exposures.

Saha et al. (2021), that exposed doxorubicin-resistant angiosarcoma cells to combinations of propranolol and doxorubicin, found a decreased cellular viability when cells were exposed to propranolol alone but when combined it increased the sensitivity of the

cells to the antineoplastic drug. Pasquier et al. (2011) that exposed six cancer cell lines (breast, non-small cell lung carcinoma, neuroblastoma and glioblastoma) and three normal cell lines to propranolol and combinations with cytostatic drugs (5-fluorouracil or paclitaxel), propranolol alone reduced viability in all cell lines and when combined, depending on the cell line and concentration of drugs, had a synergetic, antagonistic, or additive effect. Rico et al. (2017) exposed breast cancer cell lines to a combination of propranolol and metformin reporting a decrease in proliferation, mitochondrial activity, migration, and invasion. Combinations of propranolol and vincristine showed tumour regression and inhibition of angiogenesis in neuroblastoma cells (Pasquier et al., 2013).

Cohort studies suggest that β-blockers may help to improve overall survival and decrease cancer progression, distant metastases, cancer recurrence and cancer-specific death, on prostate (Grytli et al., 2013; Lu et al., 2015; Posielski et al., 2021; Zahalka et al., 2020), ovary (Diaz et al., 2012; Watkins et al., 2015), breast (Powe et al., 2010), head and neck, esophagus, stomach and colon (Chang et al., 2015) cancer. However, specificities from each group should also be considered, for example what other pharmaceuticals are being taken.

5. Conclusion and Future Perspectives

Overall, non-selective β-blockers (carvedilol and propranolol) showed higher cytotoxic effects than β1-blockers (atenolol and metoprolol) in all cell lines. Also, all pharmaceuticals cytotoxicity increased in a time-dependent manner. 22Rv1 was the most sensitive cell line to carvedilol, propranolol, and cisplatin and PC3 the most resistant cell line. The binary mixtures showed that at lower concentrations propranolol has a protective effect on PNT-2 (normal cell line), while for the same concentrations, the cytotoxic effects of cisplatin to the prostate cancer cell line 22Rv1 were increased. In conclusion the data of the present dissertation support that β-blockers can have promising effects on cancer cell lines, with minimum impact on normal cells.

Considering the data obtained in this study, future studies should be performed. One potential study should consider the use of ternary mixtures of the tested substances and other potential substances. Similar combination studies with carvedilol should also be considered as cohorts studies suggest a potential protection of this drug in cancer patients. Other compounds used for other conditions, such as the anti-diabetic metformin, or other cytostatic drugs (e.g., taxanes) should be evaluated for the treatment of this cancer. Using

the combination of drugs used in this study, other studies should explore the mechanisms underlying the elicited responses obtained at low concentrations of propranolol. The used of continued exposure under low concentrations should also be explored.

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Supplementary Information

Supplementary Table 1: Statistical comparison (paired one-way ANOVA) between the viability curves of the different time-points for the different cell lines and pharmaceuticals.

	PNT-2	22Rv1	LNCaP	PC3
Atenolol	**0.0003**	0.0528	**0.0097**	0.0907
Carvedilol	**0.0055**	0.4308	0.3993	**0.0028**
Cisplatin	**0.0039**	**0.0387**	**0.0212**	**0.0031**
Flutamide	**0.0009**	**0.0029**	0.0946	**0.0046**
Metoprolol	**0.0002**	0.0949	**0.0086**	**< 0.0001**
Propranolol	**0.0100**	0.1278	0.4498	**0.0012**

Supplementary Table 2: Statistical comparison (paired t-test) between plates with or without media renewal at 48 h for the different cell lines and pharmaceuticals tested.

48 h vs 48 h with media renewal	PNT-2	22Rv1	LNCaP	PC3
Atenolol	0.0679	**0.0108**	**0.0460**	0.4987
Carvedilol	0.0652	0.0790	0.7505	0.2930
Cisplatin	0.7986	**0.0329**	**0.0420**	0.3091
Flutamide	**0.0278**	**0.0022**	0.1004	0.7163
Metoprolol	**0.0046**	**0.0146**	**0.0168**	0.0570
Propranolol	0.0861	0.8464	0.0790	0.8848

Supplementary Table 3: Statistical comparison (paired t-test) between plates with or without media renewal at 72 h for the different cell lines and pharmaceuticals tested.

72 h vs 72 h with media renewal	PNT-2	22Rv1	LNCaP	PC3
Atenolol	**0.0048**	**0.0119**	**0.0153**	**0.0407**
Carvedilol	0.2333	0.1182	**0.0381**	**0.0199**
Cisplatin	0.0516	**0.0075**	**0.0016**	**0.0075**
Flutamide	0.7795	0.0737	0.6619	0.1139
Metoprolol	**0.0015**	**0.0200**	**0.0268**	**0.0007**
Propranolol	0.1697	**0.0355**	0.5291	**0.0019**

Supplementary Table 4: Statistical comparison (paired one-way ANOVA) between the viability curves of the different cell lines at different time-points and pharmaceuticals.

	24 h	48 h	72 h
Carvedilol	**0.0054**	0.3995	**0.0399**
Cisplatin	0.4389	**0.0308**	**0.0046**
Flutamide	**0.0003**	0.1610	0.1116
Propranolol	**0.0015**	**0.0042**	**0.0042**