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# Influence of antiparasitic benzimidazole derivatives in the cytoskeletal organization of metastatic breast cancer cells

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# Influence of antiparasitic benzimidazole derivatives in the cytoskeletal organization of metastatic breast cancer cells

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Dissertação do 2º Ciclo de Estudos Conducente ao Grau de Mestre em Química Farmacêutica

Trabalho realizado sob orientação do Professor Doutor Jorge Manuel Moreira Gonçalves e da Coorientadora Professora Doutora Paula Maria Façanha Cruz Fresco.

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É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA DISSERTAÇÃO (INDICAR, CASO TAL SEJA NECESSÁRIO, Nº MÁXIMO DE PÁGINAS, ILUSTRAÇÕES, GRÁFICOS, ETC) APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE

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# Resumo

O cancro da mama consiste num grupo heterógeno de doenças que apresenta elevada prevalência e mortalidade em mulheres a nível mundial. Estudos sobre estas doenças são fundamentais para compreender as suas causas, fatores de risco, métodos de prevenção, mas também melhorar os diagnósticos e tratamentos. Alguns dos fármacos mais usados no tratamento do cancro da mama, principalmente os mais agressivos e que não respondem a terapia hormonal atuam por interferirem com a dinâmica dos microtúbulos, nomeadamente do fuso mitótico, afetando, por isso a divisão e proliferação celulares, causando morte celular. Um mecanismo semelhante poderá estar na base dos efeitos anticancerígenos reportados para alguns fármacos anti-helmínticos, como o flubendazol, mebendazol e nocodazol, devido aos seus efeitos estudados sobre os filamentos de tubulina dos parasitas.

No entanto, os filamentos celulares de tubulina, isoladamente ou em combinação com filamentos de actina, estão também envolvidos vários outros processos celulares, relevantes para a proliferação de células cancerígenas, que poderão ser novos alvos terapêuticos destes fármacos. *Tunneling nanotubes* (TNTs) são extensões citoplasmáticas frequentemente encontradas entre células cancerosas, que são cada vez mais aceites como tendo um papel importante na tumorigénese, estando envolvidos nos processos de resistência aos tratamentos contra o cancro.

O objetivo principal deste trabalho foi o desenvolvimento e validação de um método que permita avaliar o efeito de fármacos que interferem com o citoesqueleto.

Os resultados obtidos permitem apoiar a hipótese de os TNTs poderem ser um potencial alvo farmacológico de fármacos antimitóticos, bem como de anti-helmínticos derivados do benzimidazol que mostraram ser potenciais candidatos para reaproveitamento de fármacos em oncologia.

**Palavras-chave**: cancro da mama; reposicionamento de fármacos; *tunneling nanotubes*

# Abstract

Breast cancer is an heterogenous group of diseases that presents high prevalence and mortality in women around the world. Studies on these diseases are fundamental for the understanding of their causes, risk factors, prevention methods and also for improving diagnosis and treatment. Some the anti-cancer drugs most used in breast cancer therapy, mainly in breast cancers that are more aggressive and non-responsive to hormonal therapies act by interfering microtubule dynamics, namely those present in the mitotic spindle, affecting, therefore, cellular division and proliferation leading to cell death. A similar mechanism can be the basis of the reported anti-cancer effects of some anthelmintic drugs such as flubendazole, mebendazole, and nocodazole due to their known effects on the parasite's tubulin filaments.

However, cellular tubulin filaments, alone or in combination with actin filaments, are also involved in several other cellular processes, relevant to the proliferation of cancer cells, which can become new pharmacological targets of these drugs. Tunneling nanotubes (TNTs) are cytoplasmic extensions frequently found bridging cancer cells, which are increasingly accepted as contributing to tumorigenesis and being involved in the processes of resistance to cancer treatments.

The purpose of this work is the development and validation of a method that will allow to investigate the effects of drugs known to interfere with the cell cytoskeleton.

The results obtained support the hypothesis that TNTs can be a potential pharmacological target for antimitotic drugs and anthelmintics drugs, such as benzimidazole derivatives, which have been shown to be potential candidates for drug repurposing in oncology.



**Keywords**: Breast cancer; repurposing drugs; tunneling nanotubes

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# Abbreviations

| | |
|---|---|
| **ABZ** | Albendazole |
| **BCIC** | Breast Cancer-initiating Cell |
| **CTR** | Control |
| **DMEM-HG** | Dulbecco's Modified Eagle's Medium-high Glucose |
| **DMSO** | Dimethyl sulfoxide |
| **EDTA** | Ethylenediaminetetraacetic Acid |
| **EGFR** | Epidermal Growth Factor Receptor |
| **EMT** | Epithelial-mesenchymal Transition |
| **ER** | Estrogen Receptor |
| **FBS** | Fetal Bovine Serum |
| **FLU** | Flubendazole |
| **GFAP** | Glial Fibrillary Acidic Protein |
| **HDI** | Human Development Index |
| **HER2** | Human Epidermal Growth Factor Receptor 2 |
| **HIF-1α** | Hypoxia-inducible factor 1α |
| **IARC** | International Agency for Research on Cancer |
| **IF** | Intermediate Filaments |
| **MBC** | Metastatic Breast Cancer |
| **MBZ** | Mebendazole |
| **MF** | Microfilament |
| **MT** | Microtubule |
| **MTOC** | Microtubule Organization Center |
| **NCZ** | Nocodazole |
| **PBS** | Phosphate-buffered Saline |
| **PR** | Progesterone Receptor |
| **PTX** | Paclitaxel |
| **ROS** | Reactive Oxygen Species |
| **SAR** | Structure-Activity Relationship |
| **TNBC** | Triple-negative Breast Cancer |
| **TNT** | Tunneling Nanotube |
| **VBL** | Vinblastine |
| **γ-TuRC** | γ-Tubulin Ring Complex |

# I. Chapter: Introduction

# 1. Introduction

## 1.1. Cancer

Cancer is a serious health problem that affects millions of people worldwide. It arises from the uncontrollable growth and dissemination of certain cells within the body[1, 2]. Usually, cell division serves to replenish the body's cells in a well-regulated manner[2]. However, when this process fails, abnormal or damaged cells emerge, and may give rise to tumors. Cancer cells can invade normal tissue and spread through the circulatory and lymphatic system to other areas of the body, this process being called metastasis[3].

Cancer is a numerous group of diseases that develop at the cellular level through several stages, involving mutation and selection of cells with increasing capacity for proliferation, survival, invasion, and metastasis[4]. According to the genetic hypothesis, the initiation phase of cancer development is primarily driven by genetic alterations in essential genes, including pro-oncogenic genes like *RAS* and *MYC*, as well as tumor suppressor genes such as *BRCA1, BRCA2*, and *TP53*. These mutations disrupt vital biochemical signaling pathways, affecting crucial cellular processes such as proliferation, survival, and differentiation[5-7]. Such mutations may arise spontaneously or be induced by exposure to carcinogens, broadly categorized into genotoxic or non-genotoxic[8]. While genotoxic carcinogens directly damage DNA either by themselves or through reactive metabolites, non-genotoxic carcinogens influence cancer development by altering factors like oxidative stress, metabolic enzyme function, and the balance between cellular growth and programmed cell death[8-10]. Subsequent cancer formation steps include: i) accumulation of actively proliferating preneoplastic cells (hyperplasia); ii) phenotypic changes (dysplasia) and additional genotypic alterations, enhancing invasive and metastatic potential and iii) metastasis where cancer cells spread from the primary site to other body parts *via* the circulatory or lymphatic systems[11].

### 1.1.1. Breast cancer

From a molecular point of view, breast cancer is a heterogeneous disease, and stands out as a significant health problem especially among women, given its wide occurrence worldwide[12, 13]. The latest revelation from the International Agency for Research on Cancer (IARC), through GLOBOCAN 2020 data, highlights cancer's alarming incidence and prevalence in 185 countries[14].

The global incidence of breast cancer is increasing from 2.1 million cases in 2018 to 2.3 million in 2020[14, 15]. In more developed countries (higher human development index (HDI)), this can be ascribed to reproductive/hormonal factors and lifestyle (alcohol, weight, inactivity)[14]. These trends are further amplified by the improved detection facilitated by organized or opportunistic mammography screening initiatives in those countries[14].

Despite the highest increase in the occurrence of breast cancer in developed countries, mortality rates are comparable to those observed in developing countries, as shown in **Figure 1**. (Blue bars: incidence; red bars: mortality)[14].

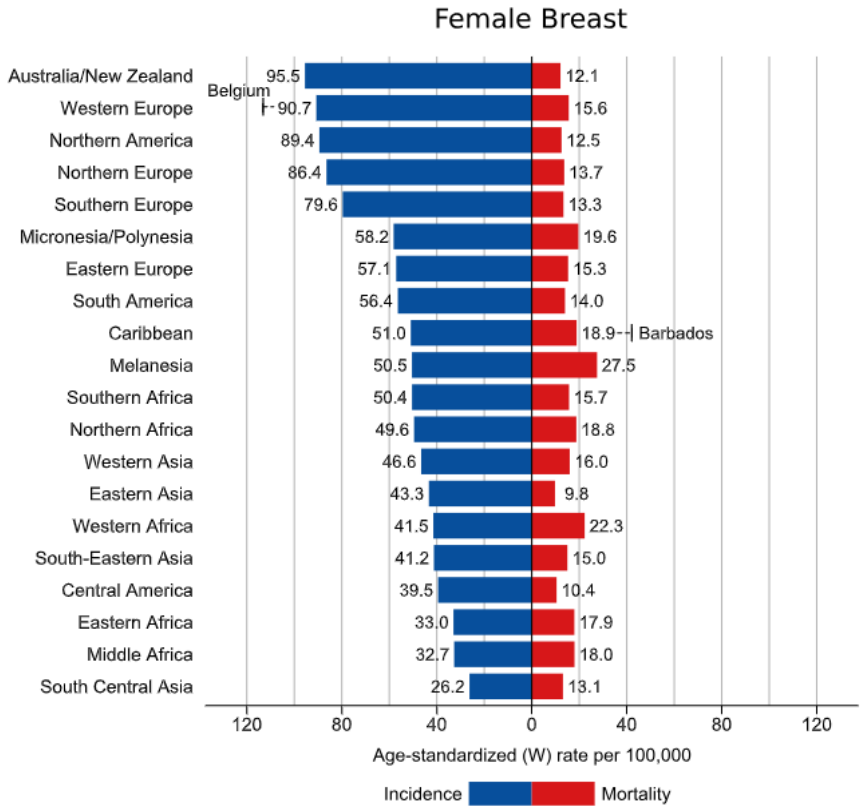



**Figure 1**: Region-specific incidence and mortality age-standardized rates for cancers of the female breast in 2020. Retrieved from[14].

Breast malignancies primarily originate from epithelial cells within the breast's glandular milk ducts and lobules, specifically in the terminal duct lobular units[16].

The breast's glandular tissue comprises a complex ductal-lobular system consisting of approximately 15 to 20 lobes interconnected by collecting ducts that converge towards the nipple[17, 18]. These lobes are further divided into smaller lobules containing acini[18]. In conjunction with the terminal duct, the acini form the functional secretory units of the

mammary gland, known as the terminal duct lobular units, where breast carcinomas typically originate[18].

The stroma encases all these structural elements, a composite mixture of fat, connective tissue, blood vessels, nerves, and lymphatics[19]. This intricate architecture forms the foundation for breast function and plays a pivotal role in developing breast malignancies[20]. **Figure 2** displays the breast's structure and highlights the specific site where certain pathological conditions may manifest.

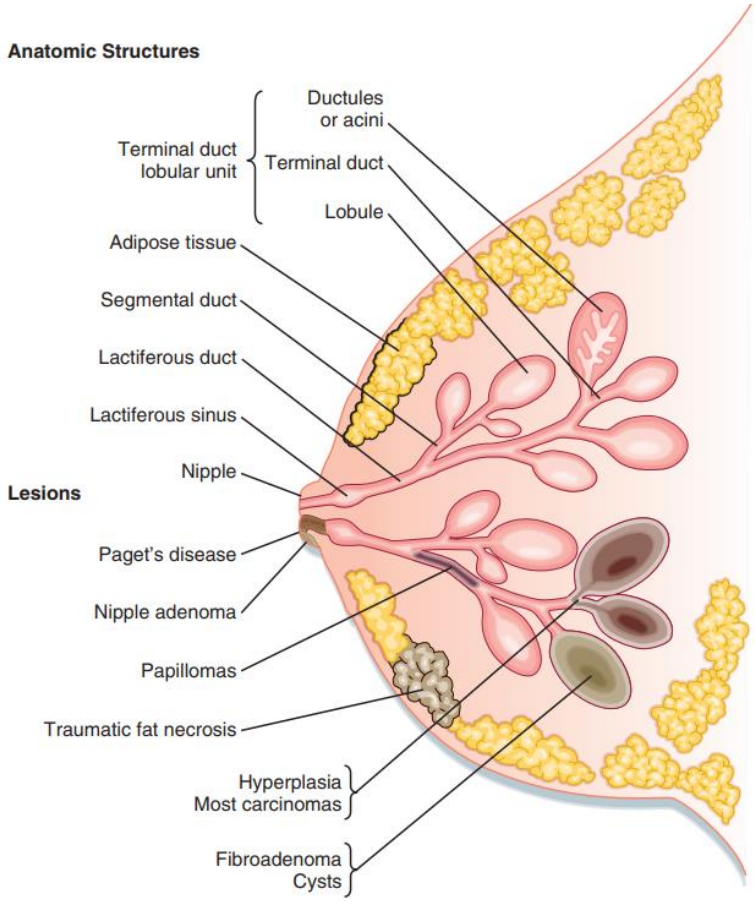



**Figure 2**: Structure of the breast and its correlation with the development of various diseases, including tumors. Retrieved from[21].

In 2000, the intrinsic classification was adopted for the first time, identifying four breast cancer subtypes: luminal A, luminal B [both expressing the estrogen receptor (ER)], basal-like, and human epidermal growth factor receptor 2 (HER2)-enriched (lacking ER expression)[22]. Presently, clinical practice frequently employs a surrogate classification system consisting of five subtypes based on both histological and molecular

characteristics[13, 23], as depicted in **Table 1**. In breast cancer classification, tumors that express ER and/or progesterone receptors (PR) are grouped under hormone receptor-positive breast cancers. Tumors lacking the expression of ER, PR and HER2 are classified as triple-negative breast cancer (TNBC)[13].

**Table 1**: Subtypes of breast cancer based on the molecular profiles. Retrieved from[23].

| Subtypes | Immunoprofiles |
|---|---|
| **Luminal A** | $ER^+$, $PR^{+/-}$, $HER2^-$ , Low Ki67 |
| **Luminal B** | $ER^+$, $PR^{+/-}$, $HER2^+$, High Ki67 |
| **Basal** | $ER^-$, $PR^-$, $HER2^-EGFR^+$, High Ki67, |
| **Claudin-low** | $ER^-$, $PR^-$, $HER2^-$ E-cadherin, claudin-3, claudinin-4 and 7 low |
| **HER2** | $ER^-$, $PR^-$, $HER2^+$ |

HER2: Human Epidermal Growth Factor Receptor 2; PR: Progesterone Receptor; EGFR-Epidermal Growth Factor Receptor

Given the diversity of breast cancer subtypes, both the prognosis and the therapeutic approach to be used will be distinct in treating breast cancer[13]. The following are the drugs currently used in chemotherapy, **Figure 3**, for treating different types of breast cancer based on their subtype and mode of action.

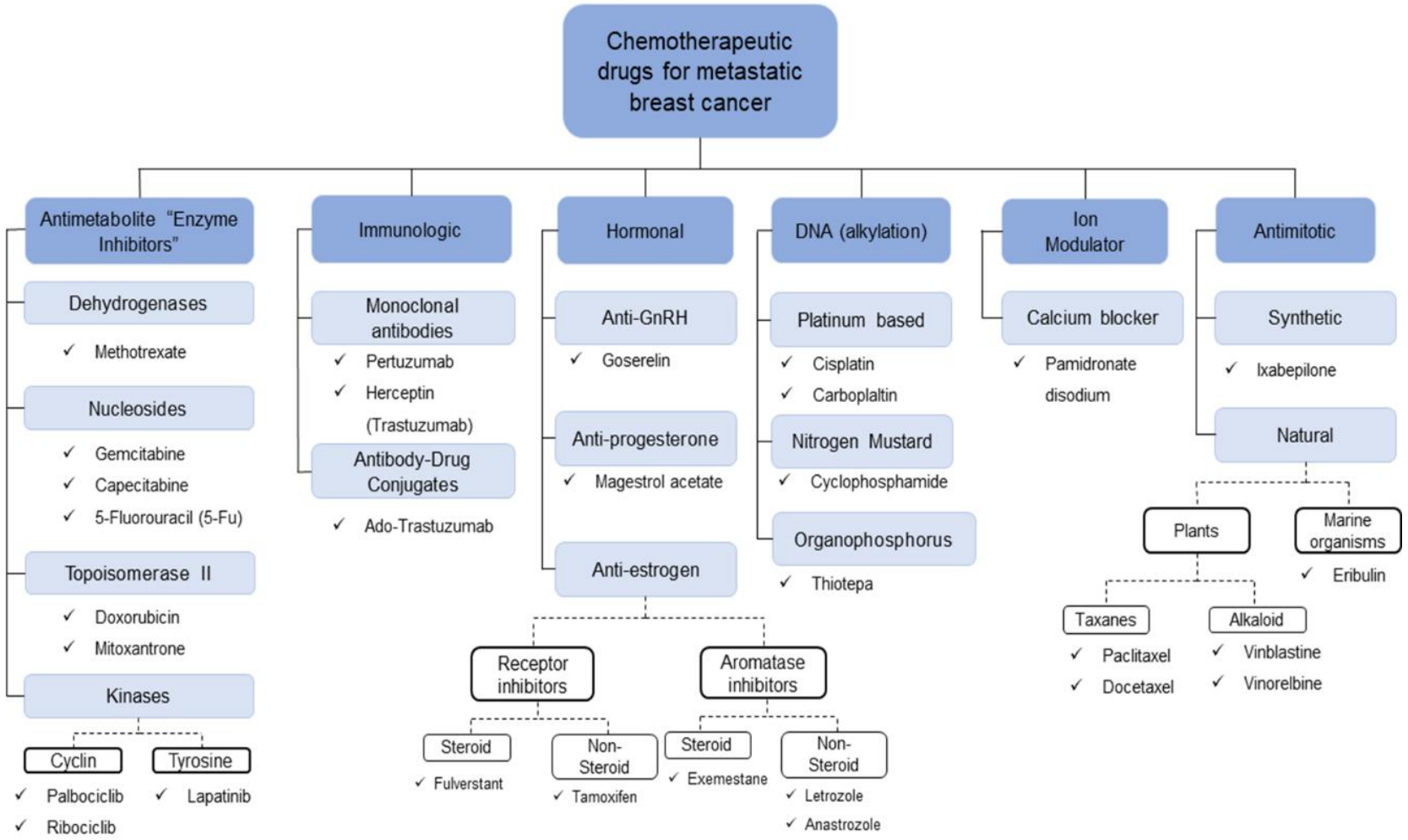



**Figure 3:** Chemotherapy drug classes for metastatic breast cancer based on mechanism of action. Adapted from[24].

Antimetabolite agents interact with vital enzymes in cells, resulting in the inactivation of these enzymes or the production of non-functional products. These agents are effective in rapidly dividing cells and are classified based on their target enzymes, such as dehydrogenases and kinases[25, 26]. The immunological therapeutic strategy focuses on tumors overexpressing HER2 (around 20% of MBC). This type of cancer is difficult to treat[27]. Activation of the receptor tyrosine kinase system generates a series of reactions that affect cell proliferation, angiogenesis, and other aspects[28, 29]. In the case of endocrine therapy, it is the main approach for most metastatic breast cancers (MBCs) that have positive hormone receptors[30]. The ER is crucial in mediating gene transcription that influences cell proliferation and invasion[30, 31]. Therapy prevents estrogen from binding to these receptors or deregulates estrogen synthesis[30, 31]. DNA alkylating agents are chemotherapeutic drugs that prevent DNA replication by intercalating between its bases, leading to cell cycle arrest[24]. Disodium pamidronate, a type of bisphosphonate, is used for palliative treatment in cases of bone metastasis in breast cancer[32]. These bisphosphonates normalize calcium levels in serum and urine and reduce lesions caused by metastases[32]. Finally, the antimitotic class is the most successful targeted therapy among the drugs presented in **Figure 3**. This class of drugs inhibits the functions of microtubules, mainly in cell division, interrupting the G2/M phase of the cell cycle and the division of cancer cells[24]. These drugs interfere with the cell cycle since the mitotic spindle is a structure made of microtubules formed during mitosis and responsible for the segregation of chromosomes in daughter cells[33]. Of this class of drugs, taxanes stand out as the preferred choice for managing patients with MBC, as evidenced by studies[24, 34]. It's worth noting that vinca alkaloids also have utility in this context. Among these alkaloids, vinblastine has found more extensive application in treating conditions such as testicular carcinoma and various lymphomas, encompassing both Hodgkin's and non-Hodgkin's lymphomas[35].

## 1.2. Cytoskeleton

The cytoskeleton, present in both eukaryotic and prokaryotic cells, consists of a complex and dynamic network of interconnected protein filaments and motor proteins that regulate various cellular functions[36, 37].

The cytoskeleton structure gives the cell its shape and helps organize the cell's parts. In addition, they provide a basis for movement and cell division. Functionally, the cytoskeleton allows the cell to adapt its shape in response to several stimuli, allowing the cell to move

through the tissues, control stiffness, adhesion, and respond to cell stress and a crucial role in cell division and, therefore, proliferation[38, 39]. These functions have implications for several physiological and pathophysiological conditions, including cancer.

The cytoskeleton is a complex network of protein fibers. It comprises three main types of proteins: actin, tubulin, and various proteins that form the intermediate filaments. These proteins are responsible for forming the primary components of the cytoskeleton - microfilaments, microtubules, and intermediate filaments - although their contributions may vary among these filament types[37]. The interactions between these components regulate the structural organization of animal cells[40]. A more detailed description of each type of cytoskeleton filaments is presented below.

### 1.2.1. Microfilaments (MFs)

MFs play an essential role in some cellular functions such as movement, intracellular transport, and cell division; they also contribute to signaling through a network of cross-communication between various proteins and signaling molecules[37, 41].

MFs are made up of actin polymers. Actin is particularly abundant in some motile cells. Actin can be presented in monomeric, globular (G-actin) or polymeric, filamentous (F-actin) forms[37]. These structures are formed when actin monomers (G-actin) associate with actin-binding proteins, thus forming two colloidal strands of actin (F-actin) in an ATP-dependent process (**Figure 4**)[37]. Briefly, the formation of microfilaments starts with binding of G-actin to the actin monomers, G-actin, forming stable dimers and the repetition of this process allows further association of G-actin to the filament, its elongation and formation of polymeric F-actin filaments[37, 38]. Polymeric actin filaments always have ATP at one end. The “plus” end. It is the end where further G-actin association with the filament may occur. The absence of ATP at the other end (the “minus” end) does not allow filament growth, and G-actin dissociation (filament disassembly) may even occur[38].

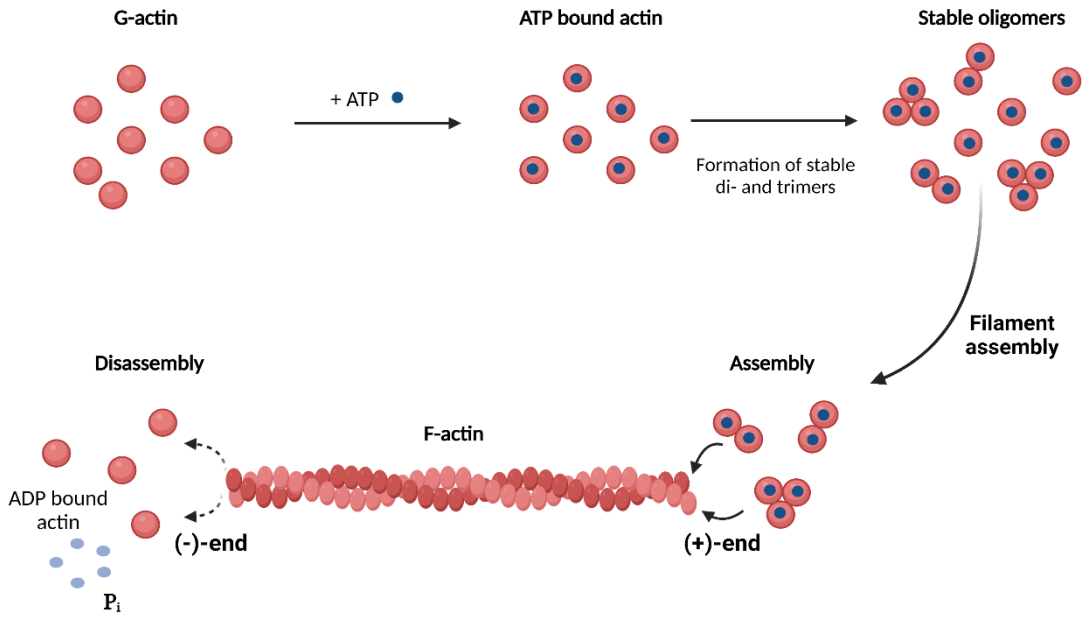



**Figure 4**: Actin filament formation. G-actin binds to ATP and forms stable di- or trimers. Finally, filaments elongate by addition of ATP bound actin monomers. Created by BioRender.com. Adapted from[38].

### 1.2.2. Microtubules (MTs)

MTs are a type of cytoskeleton filaments composed of heterodimers of alpha (α) and beta (β)-tubulin, assembled into linear protofilaments[38, 41]. MTs are initiated (or nucleated) at the microtubule organizing center (MTOC). The MTOC contains γ-tubulin, which forms the γ-tubulin ring complex (γ-TuRC) an essential structure for the initiation of the microtubules, that also works as an adaptor and anchoring proteins to stabilize and anchor the newly formed microtubules to the MTOC[42]. The inclusion of adaptor and anchoring proteins ensures that the nucleated microtubules remain attached to the MTOC and are properly oriented, allowing them to carry out their roles in the cell effectively[38].

A single MT contains thirteen protofilaments composed of α and β tubulin heterodimers that bind together to form a 25 nm wide hollow cylinder[38, 41, 43], as depicted in **Figure 5**. MTs can rapidly grow (via polymerization) or shrink (via depolymerization) in size, depending on the number of tubulin molecules they contain[38].

MTs possess two distinct ends, known as the positive and negative ends[38, 44]. This polarity is central to the regulation of microtubule polymerization. Notably, γ-tubulin complexes

predominantly associate with the negative ends. Following nucleation, α- and β-tubulin subunits bind to GTP, with GTP hydrolysis occurring when associated with β-tubulin. This hydrolysis event reduces tubulin's affinity for neighboring molecules, ultimately facilitating microtubule depolymerization[38, 44]. Thus, hydrolysis of GTP, and not of ATP, plays a fundamental role in governing the dynamic instability of microtubules.

MT instability manifests as abrupt changes in growth. It is characterized by sudden growth halts and/or rapid depolymerization—a phenomenon termed 'catastrophe,' followed by a subsequent cycle of microtubule growth[38, 44]. The transition from growth to depolymerization is called 'catastrophe,' while the reverse process—from depolymerization to growth—is known as 'rescue'[38, 44]. When new GTP-bound tubulin molecules are added faster than GTP hydrolysis, a GTP cap of the positive end is retained, and microtubules continue to grow[38]. However, when the rate of polymerization decreases or when GTPase activity increases, depolymerization and microtubule reduction occur[38]. Therefore, GTPase activity exerted by proteins belonging to the family of small GTPases are critical factor in modulating microtubule dynamics[38, 44, 45].

MTs participate in several fundamental cellular functions, namely cell compartmentalization, cell mobility, and intracellular transport. MTs are also essential in cell division by their organization into the mitotic spindle[46]. The involvement of microtubules in such important cellular functions make this cytoskeleton component a target for several drugs. In fact, several drugs that are the pillar of oncologic chemotherapy can interfere with these processes[38, 43, 47].

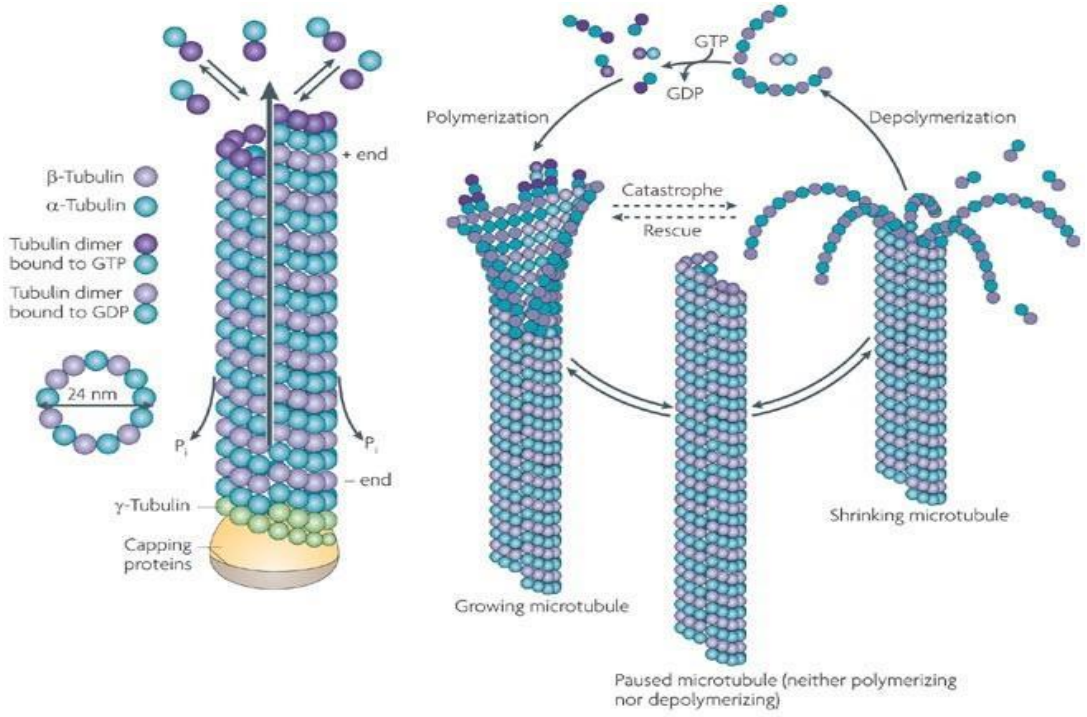



**Figure 5**: Microtubules polymerization and depolymerization. Retrieved from[48].

MTs are also important in disseminating primitive cancer cells (metastasis), a multi-step process often called the "invasion-metastasis cascade"[49].

Metastasis is a complex process, with cell motility being one of its hallmarks, as it involves cancer cell evasion and invasion[50, 51]. This process is initiated by a response of cells to an extracellular gradient of chemokines or growth factors. These factors polarize the cells and drive the extension of cell membrane protrusions towards the stimulus, a process driven by actin polymerization[41, 52].Each stage of the metastasis process requires overcoming one or more physiological barriers. These include the basement membrane and the interstitial matrix of the connective tissue, which inhibit the dissemination of malignant cells[38, 41, 53]. To overcome these barriers, cancer cells deploy proteases, enzymes that are capable of degrading the extracellular matrix, opening the path to the effects of cytoskeleton proteins that confer mobility to the cells, enabling them to resist more adverse biological environments and to disseminate in the body[41].

### 1.2.3. Intermediate filaments (IFs)

The IFs are formed through proteins encoded by at least seventy genes and organized to form filaments[38]. Depending on their structure and sequence homology, these cytoskeletal constituents can be grouped into five classes (I-V)[38].

The IFs classes are divided into filamentous: cytoplasmic intermediates (where the first four classes, I-IV, are included) and nuclear intermediates (class V, only)[38]. Cytoplasmic intermediate filaments, types I and II refer to acidic and basic keratins, respectively[38]. Their expression depends on the cell´s type and differentiation state[54]. They form heteropolymers, made up of a mixture of class I and II keratins[38]. Type III filaments are homopolymers of vimentin, expressed in fibroblasts, endothelial cells, and astrocytes; desmin expressed in muscle cells; peripherin, expressed in neurons of the peripheral nervous system; and glial fibrillary acidic protein (GFAP), expressed in astrocytes[55]. Type IV intermediate filaments consist of three neurofilament heteropolymers (NF-L/M/H): internexin, synemin, and nestin. They are expressed in cells from the central nervous system[38, 56]. To form filaments, synemin and nestin must be clustered with other intermediate filament proteins[55, 56]. Type V, referring to nuclear intermediate filaments, consists of laminins (lamin A/C, B1, B2)[38].

Structurally, the first four types of IFs are similar, consisting of a central helix with a non-helical structure at both ends[38]. Two monomers wrap around each other in a helix shape, forming a so-called “coiled-coil” dimer, followed by an antiparallel association to form non-polarized tetramers[57]. When eight tetramers are aggregated, a filament of unit length (approximately 60 nm) is formed, and these aggregate with others to form the intermediate filaments (>240 nm)[38, 58]. The mechanism of intermediate filament assembly is depicted in **Figure 6**.

In general, IFs provide mechanical force and support for cells to organize themselves[43], to maintain cell shape[43], to anchor the nucleus and some organelles in the cytoplasm and to form the nuclear lamina[59]. In the cancer context, vimentin is a notable IF as it is recognized as a key protein in tumorigenesis being overexpressed during cancer metastasis and considered a canonical biomarker of EMT (epithelial-mesenchymal transition) (EMT) and having a role in various cellular processes, including structural support, migration, and signaling[60, 61].

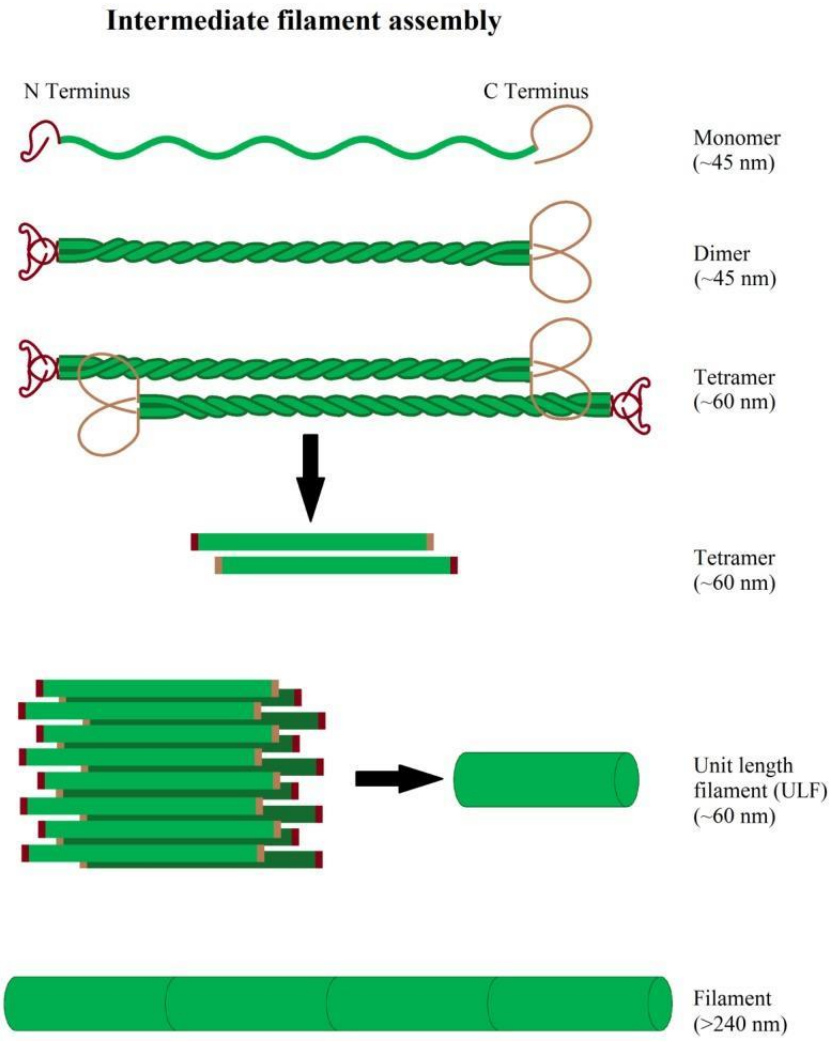



***Figure 6:*** Schematic representation of intermediate filament assembly. Retrieved from[38].

## 1.3. Tunneling nanotubes (TNTs)

Rustom and coworkers were the first authors that described these structures in the literature in 2004[62]**.** TNTs were described as long, thin and non-adherent structures formed by cytoskeleton and surrounded by plasma membrane that allow the connection between two or more cells, thus enabling cell-to-cell communication[63-65], as can be seen in the **Figure 7**. These structures have the capacity to establish cytoplasmic continuity and allow the transfer of ions, small and large molecules, and even organelles (for instance, mitochondria) between the interconnected cells[64, 66].

The role played by TNTs in different pathological states, such as neurodegenerative processes and cancer have been well documented[63]. In neurodegenerative diseases, it was shown that TNTs transport prions, and proteins such as Tau, α-synuclein, and β-amyloid[67], causing appearance of misfolded proteins in adjacent/proximal cells, and amplifying the

susceptibility to the development of neurodegenerative disorders, notably Parkinson's and Alzheimer's diseases[68-71].

In cancer, TNTs facilitate the transfer of proteins with abnormal conformation and altered genetic material[72], contributing to spread the cancer hallmarks to the non-tumorigenic cells present in the neighborhood and to the conversion of healthy cells into tumor cells[72, 73]. Communication mediated by TNTs in cancer cells has been described in various types of cancer, including pancreatic[74], prostate[75], bladder[76], several types of leukemia[77, 78], and breast cancer[79].

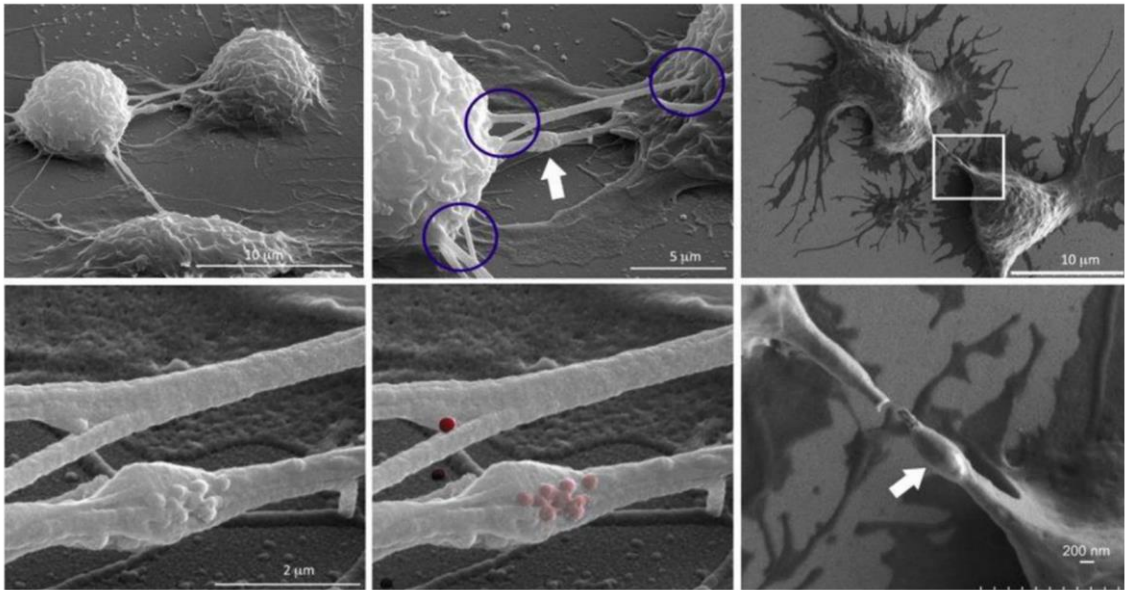



***Figure 7***: SEM images showing TNTs connecting macrophages emphasizing disparate sites of connectivity (circled) and the presence of a gondola (white arrow). Retrieved from[63].

These "bridge-like" structures are constituted by actin and tubulin surrounded by a plasmatic membrane[63]. The presence of tubulin is variable, and therefore, a distinction can be made between two types of TNTs: "thin" TNTs, constituted only by actin, of small diameter and therefore more vulnerable/fragile, and "thick" TNTs, these being constituted by actin and tubulin polymers, presenting higher diameter and, therefore, being more stable and less delicate than the previous ones[63].

The formation of TNTs can be explained by the "actin-driven protrusions" and the "cell displacement" mechanism, described in detail below[80, 81].

- **TNTs formation by actin–driven protrusions**

According to the model of actin–driven protrusions, the formation of TNTs is based on the ability of one or more cells involved in cell-cell contact to induce filopodia growth as protrusions containing F-actin[66, 80, 82]. Although the molecular basis for the formation of TNTs still remains unknown, it is conceivable that they share the same mechanism underlying the classic filopodia and their extension may be involved with the activation of the member of the Rho GTPases family, CDC42, to initiate the complex of actin nucleation[66]. Notably, in some studies, the application of actin inhibitors resulted in a significant reduction in TNT formation, providing support for the notion that actin plays a crucial role in TNT formation[83, 84].

- **Cell-dislodgement mechanism**

The cell-dislodgement mechanism states that TNTs are generated when two closely juxtaposed cells separate as they move apart[66, 82]. During this process, a residual membrane thread persists, which can be reinforced by actin, ultimately leading to the formation of TNTs[85]. According to this model, when two cells come into contact, they have the potential to form an immune synapse or undergo fusion. As the cells move in opposite directions, they give rise to a nanotube, which can originate from one or both cells involved[66, 82]. It's important to note that this mechanism is dependent on cell mobility[82].

## 1.4. Cancer drugs that interfere with the cytoskeleton

Cytoskeleton is the target of several anticancer drugs. This group of drugs are named as antimitotic drugs for their inhibitory effect on cell proliferation are being primarily attributed to their influence on microtubules during mitosis, affecting mitotic spindle formation[86].

Because of the filament diversity and because drugs may act in the same filament but at different sites, antimitotic drugs may act on the cytoskeleton in multiple ways[38]. For example, among the class of microtubule destabilizing drugs[87], drugs may act on microtubules at three distinct β-tubulin binding sites: the vinca domain, the taxane site, and the colchicine site[87]. The position of the vinca domain corresponds to the positive interface of the exchangeable GTP binding site on β-tubulin[87]; the taxane binding site is located within the microtubule's lumen in a hydrophobic pocket at the lateral interface between adjacent protofilaments[88], and the colchicine binding site is found at the intra-dimer interface between β-tubulin and α-tubulin[89].

Antimitotic drugs can be organized into two main categories: i) microtubule-destabilizing drugs, which include the vinca alkaloids (e.g. vinblastine and vincristine) and colchicine, and ii) microtubule-stabilizing drugs, the taxanes (paclitaxel and docetaxel)[86]. Drugs that destabilize microtubules interrupt the cell cycle and effect also seen with higher doses of stabilizing drugs whereas at low concentrations, both affect microtubule dynamics but do not block cell division or induce apoptosis[90, 91]. To summarize, the concentration of this type of drug determines the degree to which the cell cycle is interrupted and the drug's ability to induce apoptosis. They have more pronounced effects at higher concentrations, while at lower concentrations, they mainly affect microtubule dynamics[90, 91].

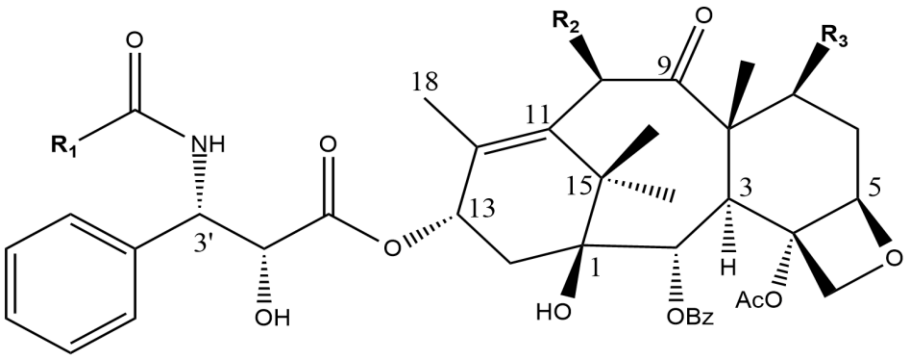
Below, the drugs in study and respective mechanisms of action will be presented. The list includes drugs used currently in chemotherapy particularly in breast cancer. It also includes drugs approved for clinical use in non-oncological indications that may interfere, with the cytoskeleton.

### 1.4.1. Microtubule stabilizing agents

Microtubule stabilizing agents encompass diverse molecules derived from various natural sources, including microorganisms, sponges, and higher plants[92]. Taxanes represent this class of microtubule-stabilizing drugs, with paclitaxel and docetaxel being the most studied[93, 94]. Paclitaxel, known by its trade name Taxol, was the first microtubule-stabilizing drug discovered. It was isolated in 1960 from the bark of the *Taxus brevifolia* tree[95, 96]. Docetaxel, on the other hand, was found after twenty years, in 1980. It is a semi-synthetic derivative of paclitaxel[97].

Structurally, paclitaxel and the semi-synthetic analogue docetaxel are very similar. They differ only in two functional groups, as can be seen in **Figure 8**. Paclitaxel consists of a baccatin core (precursor molecule of this compound), a bulky ester chain at C13, and the following relevant functional groups: hydroxyl group at C7, an acetyloxy group at C10, and in position C3' a benzyl amide[98, 99].

Docetaxel differs from paclitaxel in two positions: in the C10 position, where the acetate ester is replaced by a hydroxyl group, making this compound more soluble in water, and in the C3' position, it presents a t-butyl carbamate instead of the benzyl amide present in paclitaxel[99].




**Paclitaxel:** $R_1$= Ph, $R_2$= OAc; $R_3$=OH

**Docetaxel**: $R_1$= tBuO, $R_2$= OH; $R_3$=OH

**Figure 8**: Structures of paclitaxel and docetaxel. Structures drawn using ChemDraw Ultra 12.0.

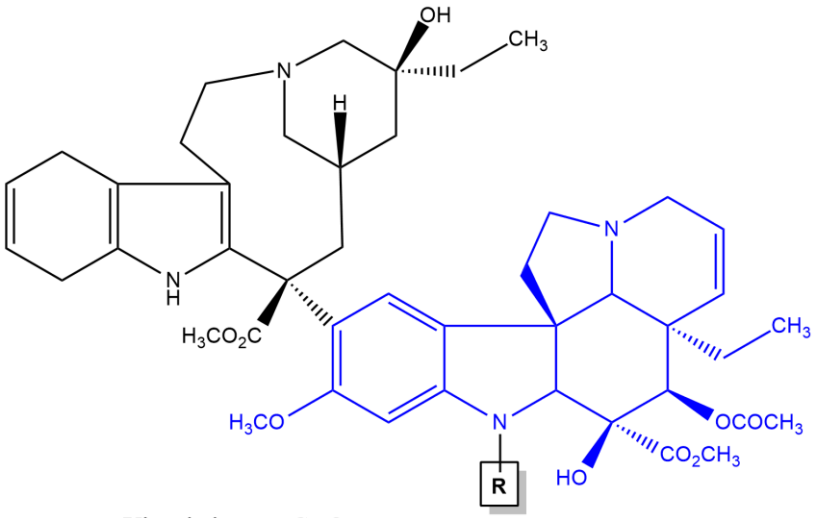
Regarding the mechanism of action, paclitaxel and docetaxel differ in some pharmacological properties, despite their structural similarities. Both drugs bind to β-tubulin, influencing microtubule polymerization and repressing the cell cycle at the G2/M phase[100, 101]. However, docetaxel has a greater tubulin binding affinity and a greater activity to inhibit microtubule depolymerization[100, 101]. When tested for antitumor activity in *in vitro* and *in vivo* models, docetaxel has a more potent antitumor activity than paclitaxel[101, 102]. Furthermore, docetaxel can reduce the expression of the anti-apoptotic gene *BCL2* which is often overexpressed in cancer cells and somehow influence microtubule dynamics[103], an effect of docetaxel than can contribute to its anti-tumor effect[100, 104].

Paclitaxel and docetaxel are used in chemotherapy and are used in the treatment of various types of cancer, namely, non-small cell lung cancer, ovarian cancer, Kaposi's sarcoma, prostate cancer and breast cancer[99, 105-108].

### 1.4.2. Microtubule destabilizing agents

Vinca alkaloids are a class of organic compounds, derived from plants, discovered in the 1950s[86, 109]. These compounds consist primarily of hydrogen, oxygen, carbon, and nitrogen[109]. Vinca alkaloids are nitrogenous bases sourced from the *Catharantus roseus G. Don* plant, commonly known as periwinkle, or be produced semi-synthetically[86, 109]. This class of compounds is renowned for its anticancer properties and finds application in various anticancer treatments[86]. Representative drugs of this group include vinblastine, vincristine, vinflunine, and vinorelbine[109].

Vinca alkaloids possess a distinctive chemical structure comprising two fundamental multi-ringed components: an indole nucleus known as catharanthine and a dihydroindole nucleus called vindoline[110]. These units are linked to other intricate systems[110]. Among the representative drugs in this class, vinblastine and vincristine exhibit remarkable structural similarity, differing primarily in a substitution within the vindoline core[110]. Vinblastine contains a methyl group in this region, whereas vincristine features a formyl group[110], as depicted in **Figure 9**. Notably, this subtle structural variation does not alter the mechanism of action or the tubulin-binding properties of these drugs but give rise to differences in their antitumor and toxicological profiles[111].




**Vincristine:** R=CHO

**Vinblastine**: R=$CH_3$

**Figure 9:** Structures of vinblastine and vincristine, vindoline (black), and catharanthine (blue). Structures were drawn using ChemDraw Ultra 12.0.

The mechanism of action of vinca alkaloids has yet to be fully understood, given the wide range of their biochemical effects on cells and tissues. It is known that these drugs act at the microtubule level, preventing cell growth and leading to apoptosis[86, 112].

A microtubule has between sixteen and seventeen binding sites located at the ends of microtubules, where these drugs can bind, interrupting the agglomeration of microtubules, shortening microtubules, and inhibiting their function[112, 113]. Inhibition of micro-spindle formation is the most often described effect of these drugs, causing the dissolution of the mitotic spindle and the arrest of the cell cycle at metaphase/ M phase[86, 113, 114]. The disintegration of the mitotic spindle causes the accumulation of chromosomes in unnatural forms (balls and stars), leading to cell death by activating apoptotic pathways[113]. Inhibition of micro-spindle formation has been the proposed mechanism to explain the anticancer activity of these drugs[86, 109, 113].

Vinca alkaloids have been proven to be highly effective in treating certain types of cancer, such as lymphatic and hematological neoplasms, as well as solid tumors, like non-small cell lung and breast cancers[109, 114]. These compounds can be used on their own, but they can also be combined with other drugs for even greater efficacy[109].

## 1.5. Repurposed drugs and cytoskeleton

In addition to the aforementioned drugs that are already being used as anticancer agents, several other drugs, initially approved for clinical use in non-cancer applications, have been found to interfere with cytoskeleton structure. Such drugs are termed "repurposed drugs." Drug repurposing is an approach that seeks to discover new therapeutic uses for already approved drugs. This strategy is garnering significant interest as it holds the promise of unveiling new indications beyond those initially approved[115].

### 1.5.1. Anthelmintic drugs

Some anthelmintic agents function by inducing paralysis in parasites, thereby allowing the host's immune system to either eliminate the parasite or alter its metabolism. Human-infesting helminths are typically categorized into three groups: nematodes (worms), trematodes, and cestodes (tapeworms)[116, 117]. It's important to note that parasite metabolism exhibits significant variations across species. This implies that a specific drug may

effectively target one species but have no impact on another[117]. This highlights the need for tailored treatments designed to address specific parasitic infections.

Anthelmintic drugs can be classified into various categories based on shared chemical structures and mechanisms of action[117]. These categories encompass piperazines, benzimidazoles, levamisole, pyrantel and morantel, paraherquamide, ivermectin, emodepside, and nitazoxanide[117]. Among these classes, the benzimidazole family stands out due to its potential to disrupt the organization of the cytoskeleton[117]. Therefore, we will exclusively delve into a detailed description of some drugs of this particular group of anthelmintic agents.

Benzimidazole is characterized by the bicyclic structure, in which a benzene ring fuses at positions 4 and 5 of an imidazole ring[118, 119]. This unique structure is considered privileged and a significant pharmacophore in medicinal chemistry[120].

The benzimidazole family has been the subject of intense investigation, proving to be a class of drugs with a broad spectrum of pharmacological activities[119-122], as illustrated in **Figure 10**.

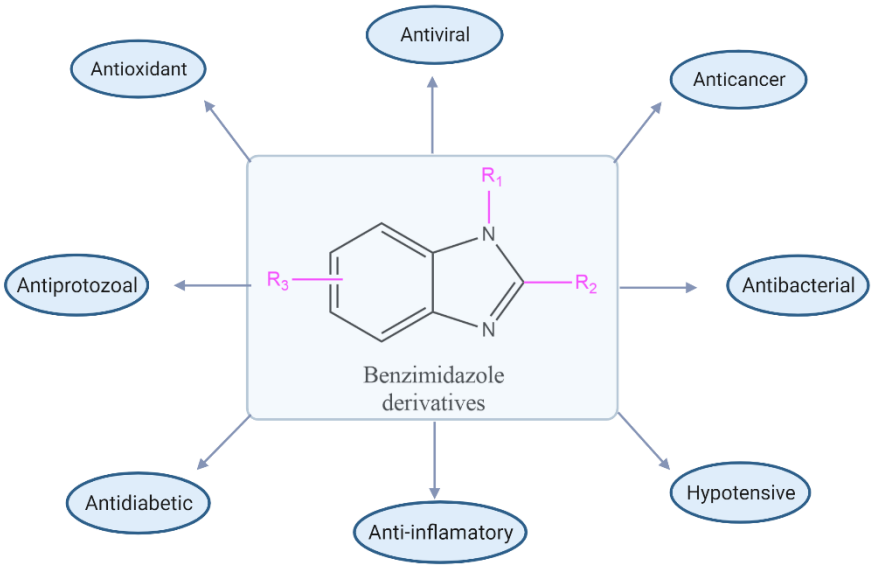



**Figure 10:** Structure of benzimidazole derivatives and their pharmacological activities. Structure drawn in ChemDraw and scheme made using BioRender.

Within the benzimidazole family, our focus narrows to drugs that, alongside their antiparasitic properties, have demonstrated noteworthy anticancer potential. Notably, among these are albendazole, flubendazole, mebendazole, and nocodazole, all classified as benzimidazole carbamates[123].

Benzimidazole carbamates share a striking structural similarity, featuring carbendazim (methyl-N-(1-H-benzimidazol-2-yl carbamate)) in their composition, as depicted in **Figure 11**. Carbendazim exhibits the capability to impede tumor cell proliferation by disrupting the cell cycle at the G2/M phase and induce apoptosis[124]. Hammond et al. have documented this effect, particularly in breast, ovarian, lung, and colon carcinoma cell lines[124].

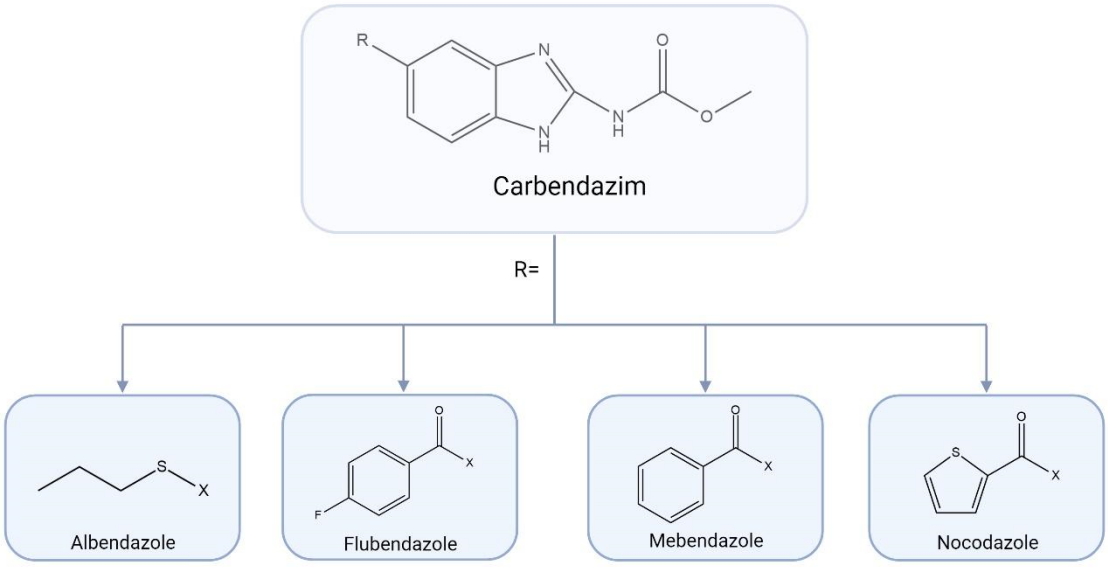



**Figure 11:** Chemical structure of carbendazim (X) and the substitutions that give rise to the drugs: albendazole, flubendazole, mebendazole and nocodazole. Structures drawn in ChemDraw.

Upon examining the chemical structures of these compounds, it becomes evident that mebendazole and flubendazole share the closest structural resemblance among the benzimidazole derivatives. As a result, their mechanisms of action and metabolic pathways exhibit similarity.

The mechanism of action for benzimidazole carbamates is well-established. It is centered on the affinity of these compounds for the parasite's β-tubulin, causing inhibition of microtubule polymerization, ultimately disrupting the cellular structure of the parasite[125]. Additionally, these drugs impede glucose uptake, leading to a depletion of glycogen reserves and a reduction in ATP formation within the parasite cells[126-128]. Consequently, these dual actions result in the immobilization and eventual demise of the parasite[126-128].

**Table 2** summarizes the cancer types tested for antiproliferative and antitumor effects, along with key findings on the mechanism of action of the benzimidazole derivatives.

**Table 2**: Mode of action of benzimidazole anthelmintics and types of cancer where anticancer activity has been found. Adapted from[150].

| Anthelmintic drug | Albendazole | Flubendazole | Mebendazole | Nocodazole |
|---|---|---|---|---|
| **IUPAC name** | Methyl [5-(propylthio)-1H-benzoimidazol-2-yl] carbamate | Methyl N-[6-(4-fluorobenzoyl) -1H-benzimidazol-2-yl] carbamate | Methyl (5-benzoyl-1H-benzimidazol-2-yl) carbamate | Methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate |
| **Cancer type** | Lung cancer[129]; gastro-intestinal (GI) cancer[130]; ovarian cancer[131]; head and neck cancer[132]; breast cancer[133]; leukemia[134]. | Breast cancer[135]; melanoma[136]; neuroblastoma[137]; leukemia and myeloma[138]. | Lung cancer[139]; leukemia[140]; brain cancer[141]; GI cancer[142]; breast cancer[143]; prostate cancer[144]; melanoma[145]. | Breast cancer; colorectal cancer[146], leukemia[147]. |
| **Results in preclinical studies** (in vivo and in vitro) on breast cancer | • Triggers G2/M phase arrest and apoptosis[133]<br>• Suppresses cell proliferation and migration[126]<br>• Stimulates ROS generation[126] | • Leads to G2/M phase detainment, activates caspases, initiates autophagy, and triggers apoptosis[148]<br>• Demonstrates efficacy against drug-resistant cancer cells[149]<br>• Provokes the differentiation of BCSC-like cells[135] | • Triggers G2/M catastrophe, causes double-strand DNA breaks, and prompts apoptosis[150]<br>• Enhances sensitivity in TNBC and hinders breast cancer-initiating cells (BCIC) formation[147] | • G2/M cell cycle arrest[151]<br>• Induces apoptosis[151]<br>• Disrupts the morphology and directional movement of migrating medial ganglionic eminence cells[151] |

With regard to the mechanism of action of these drugs as anticancer agents, it's important to note that the complete understanding of this mechanism remains elusive. However, certain authors have proposed potential mechanisms, particularly in the context of breast cancer, where these drugs may operate as follows:

Albendazole triggered the activation of autophagy and apoptosis processes while also triggering inhibition in the cell proliferation and migration rate[133]. This action was achieved through down-regulation of the AMPK/P53 signaling pathway[133], mediated by GLUT1. In malignant melanoma (A375) and breast cancer (MCF7) cells, after treatment with ABZ, a reduction in negative regulators and a consequent increase in p53 and p21 expression was observed[152]. When there is an unbalanced increase in reactive oxygen species (ROS), there is a favoring of antitumorigenic signaling, leading to apoptosis; that is, agents that generate ROS may have potential as anticancer drugs[153]. DNA damage caused by ROS triggers the activation of apoptosis, inducing histone H2AX phosphorylation and p53 and Bax expression[126].

Flubendazole triggers G2/M phase arrest, caspase activation, STAT3 impairment, and apoptosis in some triple-negative breast cancer cell lines (namely, MDA-MB-231)[148]. After treatment with this drug, less activation of STAT-3 and lower levels of MMP-2/MMP-9 were observed in vivo, demonstrating the potential to eliminate BCSC-like cells that drive resistance to therapy[148].

Concerning mebendazole, investigations into the effects of this drug on breast cancer cells have been limited due to the challenge presented by radiotherapy that leads to the transformation of breast cancer cells unaffected by radiotherapy into breast cancer-initiating cells (BCICs) resistant to therapy[154]. When triple-negative breast cancer cell lines (specifically, SUM159PT and MDA-MB-231) were treated with this drug, these cells became more sensitive to radiation[155]. Mebendazole triggers a catastrophic mitotic event, which results in double-strand breaks in DNA and subsequent apoptosis of breast cancer cells after radiotherapy[155].

Lastly, when cancer cells are treated with nocodazole, it disintegrates microtubules, preventing their binding to the kinetochores during the prometaphase phase[156]. The kinetochores that remain unattached are then anchored by the MAD2 protein, interrupting the transition from prometaphase to metaphase and anaphase[156]. The mechanism needs to be better understood, but the presence of MAD2 protein bound to kinetochores is believed to play a crucial role in upregulating cyclin B1 and Cdc2 proteins in cells stuck in prometaphase[156]. The rapid increase of these two cell cycle proteins, especially their nuclear accumulation, is likely responsible for the emergence of distinct nuclear features[156].

In situations where prometaphase is prolongedly disrupted, cells treated with nocodazole are expected to undergo apoptosis via intrinsic cell death pathways[156].

# II. Chapter: AIM

# 2. AIM

Oncological antimitotic drugs and repurposable anthelmintic drugs from the benzimidazole family share the capability to interfere with the dynamics of microtubules as part of their mechanism of action. Although the inhibition of the mitotic spindle formation is often highlighted as the primary means by which antimitotics exert their anticancer effects, understanding other roles played by the cytoskeleton urges the need to study the impact of various drugs on these effects.

TNTs, recently discovered structures, seem to play a significant role in the survival of tumor cells and in carcinogenesis, particularly in metastasis. This can be ascribed to the fact that TNTs would allow surviving cells to share resources with adjacent cells to withstand chemotherapy and radiotherapy.

The purpose of this work is to develop and validate a method to investigate the effects of these drugs on the dynamics of microtubules in these structures and, subsequently, to use this method to analyze the activity of benzimidazole-derived antiparasitic drugs on such components of the cytoskeleton.

# III. Chapter: Material and methods

# 3. Material and methods

## 3.1. Chemicals

Fetal bovine serum (FBS) was purchased from BioChrom (Biotecnómica, São Mamede, Portugal). Trypsin, Dulbecco's Modified Eagle's Medium – high glucose (DMEM-HG), and PrestoBlue™ were obtained from Invitrogen (Alfagene, Carcavelos, Portugal). Vinblastine was from Tocris Bioscience (Biogen, Madrid, Spain). Flubendazole, mebendazole, nocodazole, paclitaxel, and Hoechst 33342 were purchased from Merck/Sigma-Aldrich (Chemicalnor, Valongo, Portugal). Flash Phalloidin™ green 488 was purchased from Biolegend (Lusopalex, Lisboa, Portugal).

## 3.2. Cell culture and cell maintenance

In this dissertation, the cell line used was MDA-MB-231, an epithelial human breast cancer cell line. The cells were a kind gift from i3S - Portugal.

MDA-MB-231 cells were cultured in T25 $cm^2$ cell culture flasks with DMEM-HG medium containing 4.5g/L of glucose and supplemented with 10% FBS, 100µg/mL of streptomycin and 100 U/mL of penicillin. The cell line was maintained in a humidified atmosphere, at 37°C and with 5% $CO_2$.

For cell culture maintenance, the cells are divided twice in one week when the confluence level was close to 80%. First, the medium is removed, and the cells are washed with 100 mM PBS. Subsequently, the cells are treated with a trypsin solution (0.25% v/vtrypsin; 0.025% v/v EDTA in PBS) and incubated for six minutes at 37°C to detach the cells from the culture flask. Finally, the cells are resuspended in a new medium and placed in new culture flasks.

## 3.3. Experiments in chemically induced hypoxia conditions: determination of the best concentration of cobalt chloride ($CoCl_2$)

Hypoxia conditions occurring within tumors form oxygen gradients, increasing tumor diversity and aggressiveness, leading to heightened metastatic behaviors[157, 158]. Oxygen deficiency triggers gene expression shifts, altering protein production. These changes

impact diverse cellular functions and processes[158]. Notably, the hypoxia-inducible factor 1α (HIF-1α) plays a pivotal role, central to cellular responses to hypoxia-induced mechanisms[158]. In the experiments where chemically, induced hypoxia was caused by the addition of cobalt chloride ($CoCl_2$), it was necessary to do preliminary tests to verify the best concentration to use.

Based on the concentrations used by our group in previous works and on the existing literature on the same cells used under conditions similar to those intended, four concentrations were chosen (100 µM, 75 µM, 50 µM, and 25 µM), from which the TNTs were tested and quantified in two-time points, 24 h and 48 h. Based on the results obtained in the cell viability assay and quantification of TNTs, the most promising concentration was 25 µM after 48 hours of cell plating.

## 3.4. Cell treatment

In the conditions of normoxia, for each experiment, the MDA-MB-231 cells were centrifuged at 0.4 rcf for five minutes at 20°C, and cells were counted using the trypan blue dye exclusion method. MDA-MB-231 cells were plated with an initial cell density of 1.4 x $10^4$ cells.$mL^{-1}$ in a complete medium and incubated for 24 h (200 µl per well). After this period, the medium is removed, and cells are treated with the following drugs at different concentrations: paclitaxel (5 nM - 40 nM), vinblastine (50 nM - 5 µM), flubendazole (0.1 µM - 0.8 µM), nocodazole (25 nM - 200nM) and mebendazole (10 nM - 1 µM). Experiments were also performed to mimic hypoxia using cobalt chloride ($COCl_2$). In these experiments, cells were seeded (100 µl per well), and after the incubation time, drugs at different concentrations were added with 25 µM of $COCl_2$ (100 µl per well).

Stock solutions of the tested drugs were prepared using DMSO, and final concentrations were obtained by dilution with culture medium (DMEM-HG) moments before each experiment. The control/solvent experiments were performed by treating the cells with DMSO (0.1%) and, in the case of the hypoxia experiments, also with $COCl_2$ (25 µM). The cells are incubated at 37°C for 48h.

## 3.5. Cell viability assay

The cell viability assay used is the resazurin reduction assay (using the PrestoBlue™ reagent. The PrestoBlue™ reagent contains resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) and the assay evaluates cell viability and cytotoxicity. Viable cells, when in contact with this reagent, reduce resazurin (a blue, non-fluorescent compound) to resorufin (a red fluorescent compound), and the fluorescence intensity (excitation 530 nm, emission 590 nm) is directly proportional to cell viability[159]. Fluorescence was measured usingan automated microplate reader (Sinergy HT, Biotek Instruments Inc, Winooski, VT, USA).

## 3.6. Actin staining with Flash Phalloidin™ Green 488

MDA-MB-231 cells were fixed with 4% paraformaldehyde for 10 minutes, washed twice with PBS, cells were permeabilized with 0,5% Triton X-100 for 10 minutes and washed twice with PBS, blocked cells with 5% FBS for 30 minutes. The Flash Phalloidin™ green 488 was diluted in the proportion 1:20 – 1:100 in PBS. Next, the cells were stained with the diluted solution for 20 minutes in the dark, protected from light. After this step, the cells were also stained with Hoechst 33342 (5 µg/mL) for 30 minutes for nuclei staining. Finally, fluorescence images were acquired using the Lionheart FX using the GFP and DAPI channels.

## 3.7. Quantification of TNTs

The TNT quantification method involves analyzing images obtained using an automated inverted microscope (Lionheart FX) able to use microplates in both fluorescence and phase contrast modes. This approach allows the manual identification and quantification of TNTs based on criteria previously established in the literature[160, 161]. The specific criteria applied in this master's thesis are the following:

1. TNTs are recognized as connections between two or more cells, spanning at least 5 µm in length, which are not adhered to the substrate.

2. Membrane extensions that do not establish connections between at least two cells were not included in the TNT count.

3. In each individual experiment, different treatments were carried out in triplicates and, for each well, at least 120 to 130 cells were counted, and the experiment was independently conducted at least three times for statistical analysis.

4. Concerning TNTs' size differentiation, "thick" TNTs are identifiedas those with a diameter equal to or exceeding 0.7 µm, whereas "thin" TNTs are characterized by a diameter lower than 0.7 µm[80, 162].

Cell identification and localization were performed in fluorescence mode using Hoescht 33342 and the DAPI channel to identify stained nuclei and images were acquired. The count was based on collected images and using the Gen5 image analysis software for automatic counting. The images used for quantification were collected from the same area of each well, which allowed comparability between different experimental conditions.

The TNTs were quantified based on images captured in phase contrast mode, as already mentioned, with multiple images of each field from different planes (pseudo-Z-stack) with several images in the Z plane (Z = 0, 4, 8µm). The use images from the same field but in different planes allows better identification of TNTs, as these are non-adherent structures and, therefore, are arranged in irregular planes. As the quantification of TNTs is manual and, therefore, subject to operator decisions, two independent operators repeat this operation, and the average of the values obtained by the independent operators is considered for each sample. Whenever the count of the independent operators differs more than 15%, a third operator was involved in analyzing the counting disparities and adjusting the final value. The effect of drugs was evaluated by the ratio between the number of TNTs per cell in the presence and absence of drug (cells treated only with the same volume of solvent used to prepare the drug solution).

## 3.8. Statistics

Data were tested for normality (Shapiro-Wilk test), using the statistical software *IBM SPSS* version 29.0.0.0. The results are presented as mean ± SD of at least three independent experiments. Differences between treatments and controls were compared using Student´s *t* test, using the GraphPad Prism X V software. A value of $p<0.05$ was considered to demonstrate statistically significant differences.

# IV. Chapter: Results

# 4. Results

## 4.1. Effect of drugs interfering with the cytoskeleton on MDA-MB-231 cell viability

Initially, the experiments were executed under normoxic conditions. To understand the effect of drugs known to interfere with the cytoskeleton and present in the pharmacotherapy arsenal as anticancer (antimitotic drugs, **Figure 3**) and anthelmintics drugs, experiments were carried out to evaluate their effect on the cell viability of MDA-MB-231 cells, using the PrestoBlue™ reagent assay[163, 164]. The drugs tested were the antimitotic agents paclitaxel (5 nM – 40 nM) and vinblastine (50 nM – 5 µM) and the anthelmintics flubendazole (0.1 µM – 0.8 µM), mebendazole (10 nM – 1 µM) and nocodazole (25 nM – 200 nM). These concentrations were chosen based on the IC50 values of the drugs and studies previously carried out using this cell line[165-167].

The effect of each drug at different concentrations, under normoxia conditions, on MDA-MB-231 cell viability is presented in **Figure 12**. After incubation for 24 hours, the antimitotic drugs paclitaxel (40 nM) and vinblastine (50 nM and 5 µM), markedly reduced MDA-MB-231 cell viability (**Figure 12A**). Concerning the drugs of the anthelmintic class in study (flubendazole, mebendazole and nocodazole), only flubendazole, at the highest concentration tested (0.8 µM), was able to reduce MDA-MB-231 cell viability (**Figure 12B**). All other treatments (other concentrations of flubendazole and all concentrations of mebendazole and nocodazole) did not cause significant effects on cell viability **(Figure 12B**).

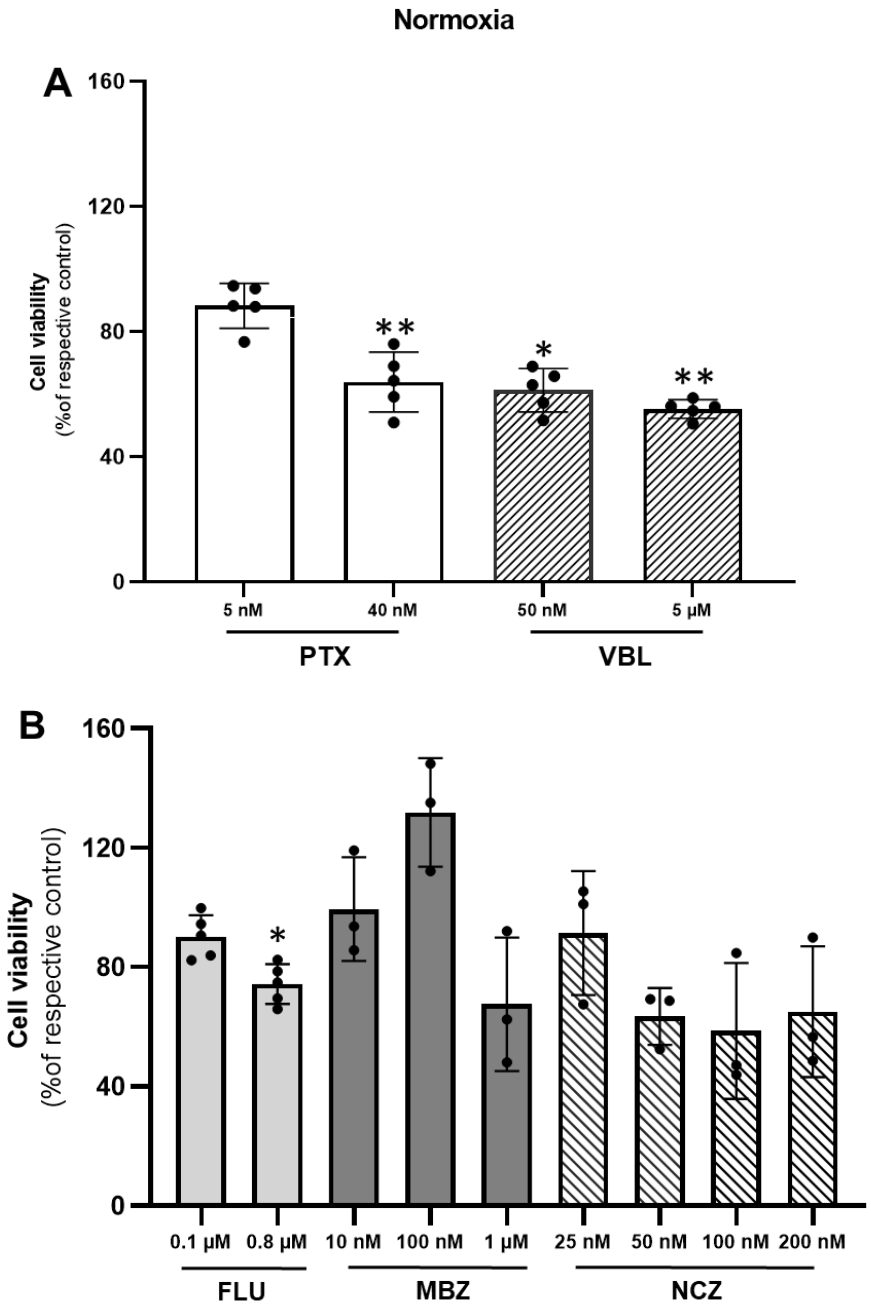



**Figure 12**: **Effect of drugs used in therapy as anticancer (A) and anthelmintic drugs (B) on MDA-MB-231 cell viability, after 24 hours incubation, under normoxic conditions.** MDA-MB-231 cells were seeded for adhesion and then treated with the indicated drugs, at increasing concentrations, for 24 hours. Cell viability was then evaluated using the PrestoBlue™ reagent assay. The results are expressed as percentage of the respective control (cells treated with DMSO 0.1%). Data from three to five independent experiments are presented as mean ± S.D. for each compound (three replicates). Statistically significant differences from the respective control: *p<0.01; **p<0.001 (Student´s *t*-test). PTX: paclitaxel; VBL: vinblastine; FLU: flubendazole; MBZ: mebendazole; NCZ: nocodazole.

## 4.2. Influence of anticancer and anthelmintic drugs on the formation of Tunneling Nanotubes (TNTs)

The drugs studied (paclitaxel, vinblastine, flubendazole, mebendazole, and nocodazole) interfere, as mentioned above, with tubulin polymerization. Since TNTs are structures made up of actin and, in some cases, tubulin, it is relevant to investigate whether these drugs can affect the formation of these structures in the treated cells. Phase contrast images, obtained using an automated inverted microscope, of the same region of the well, were used to manually identify and count these structures. The number of cells was automatically counted by counting the nuclei in the same image, using the Gen 5 Image Analysis Software (Lionheart FX). These values were then used to calculate the ratios of the number of TNTs/cell, for both control (cells incubated with the solvent, DMSO 0.1%) and treated cells (different drugs and concentrations). **Figure 13A** shows that treatment of MDA-MB-231 cells with 40 nM of paclitaxel or vinblastine at the two concentrations tested (VBL; 50 nM and 5 μM) elicited a significant decrease in the number of TNTs/cell, suggesting that the anticancer drugs affect not only microtubules dynamics but also interfere with TNTs formation.

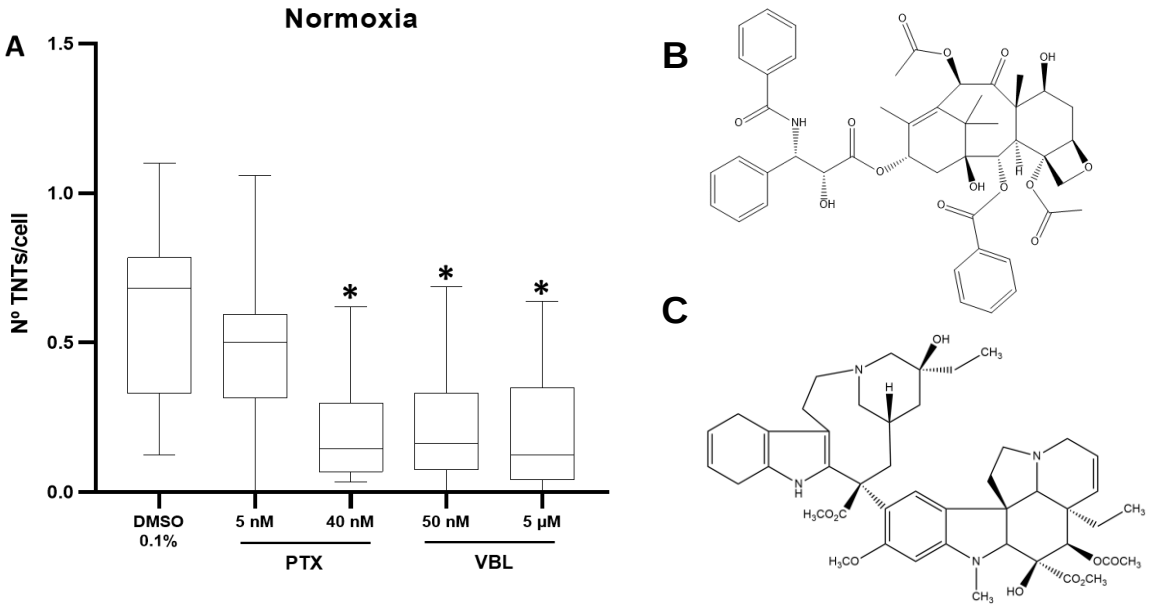



**Figure 13: Effect of PTX and VBL treatment (24h) on the number of TNTs/cell in MDA-MB-231 cells, under normoxia conditions (A). Chemical structures of PTX (B) and VBL (C).** The quantification of the number of TNTs was done manually through the analysis of phase contrast images captured by Lionheart FX. The detailed method of quantification of TNTs can be found in Materials and Methods, section 3.7. Data from five independent experiments are expressed as median and interquartile ranges of three wells for each compound (three replicates). Statistically significant differences from control: *p<0.01 (Student´s t-test). PTX: paclitaxel; VBL**:** vinblastine**.**

When MDA-MB-231 cells were treated with anthelmintic drugs, flubendazole showed a tendency for decreasing the number of TNTs/cell (**Figure 14A)** although this effect was not significant. The lowest concentrations of mebendazole (MBZ; 10 nM; **Figure 14B**), and nocodazole (NCZ; 25 nM; **Figure 14C**), also seemed to decrease the number of TNTs/cell, although, once again, these effects were not significant. Strikingly, this compound showed a biphasic effect for the other two concentrations tested: 100 nM of mebendazole significantly increased, whereas 1 μM of the same drug decreased the number of TNTs/cell (**Figure 14B**). Concerning nocodazole, all concentrations tested (50 nM, 100 nM, 200 nM) elicited significant decreases in the number of TNTs/cell in MDA-MB-231 cells (**Figure 14C**).

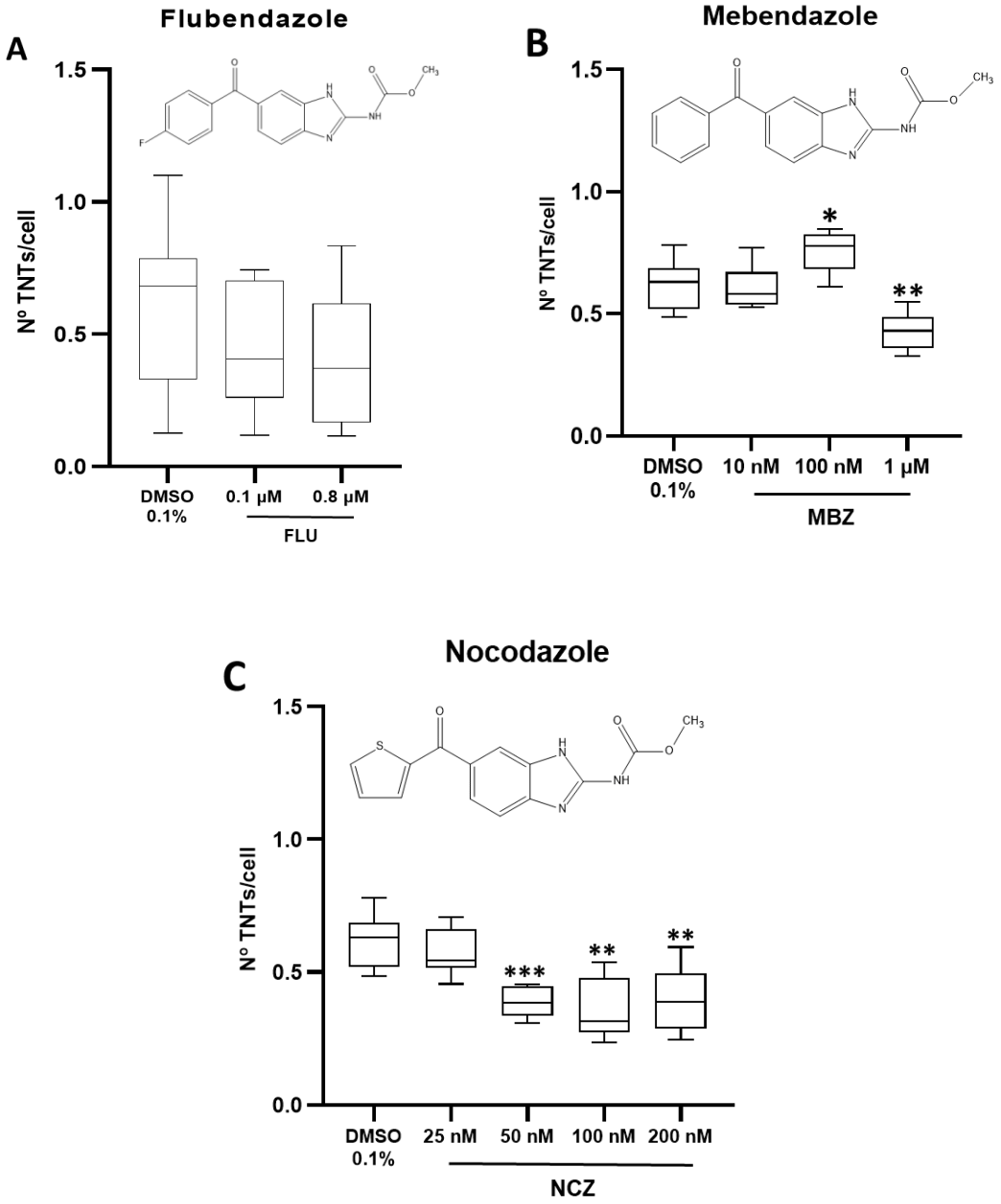



**Figure 14**: **Effect of FLU (A), MBZ (B), and NCZ (C) treatments (24 hours) on the number of TNTs/cell in MDA-MB-231 cells, under normoxia conditions.** The quantification of the number of TNTs/cell was done by analysis of phase contrast images captured by Lionheart FX. The detailed method of quantification of TNTs can be found in the section Materials and Methods, section 3.7. Data from three independent experiments are expressed as median and interquartile ranges of three wells for each compound (three replicates). Statistically significant differences from control: *p<0.05; **p<0.01; ***p<0.001. (Student´s *t*-test). FLU**:** flubendazole**;** MBZ**:** mebendazole; NCZ**:** nocodazole.

## 4.3. Influence of antimitotic and anthelmintic drugs on the size of TNTs

As mentioned above, TNTs can be categorized into two types based on their protein composition and thickness. Those composed solely of actin are termed "thin" TNTs, **Figure 15A**, whereas those containing both actin and tubulin are termed "thick" TNTs, **Figure 15B**. This differentiation is also determined by their diameter, with "thin" TNTs having a diameter of less than 0.7 µm and "thick" TNTs having a diameter equal to or exceeding 0.7 µm[63].

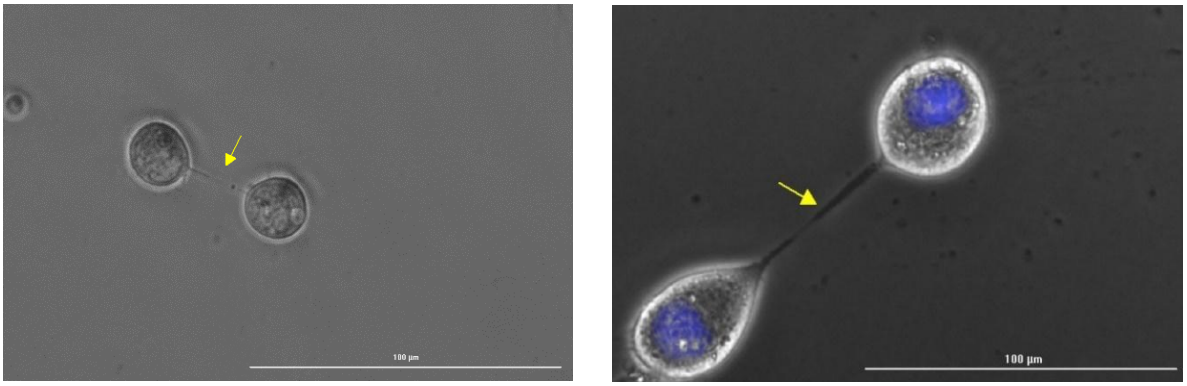



**Figure 15**: **Representative phase contrast microphotographs of TNTs identifying a "thin" (A) and a "thick" (B) TNT.** TNTs in MDA-MB-231 cells were quantified manually and TNTs were measured and distinguished according to their diameters as described in Materials and Methods section 3.7. Yellow arrows indicate TNTs. Blue fluorescence indicates nuclei stained with Hoechst 33342. Scale bar: 100 µm.

The diameter of TNTs was assessed for various drug concentrations, and using the criteria mentioned above, the number of "thin" and "thick" TNTs were counted in individual images (corresponding to individual wells/treatments).

Data presented are the relative proportions (in percentage) of the total TNT number present in both control and treated cells samples. Vinblastine (5 µM) caused a decrease in the percentage of "thick" TNTs when compared to the relative values present in control samples (**Figure 16**).

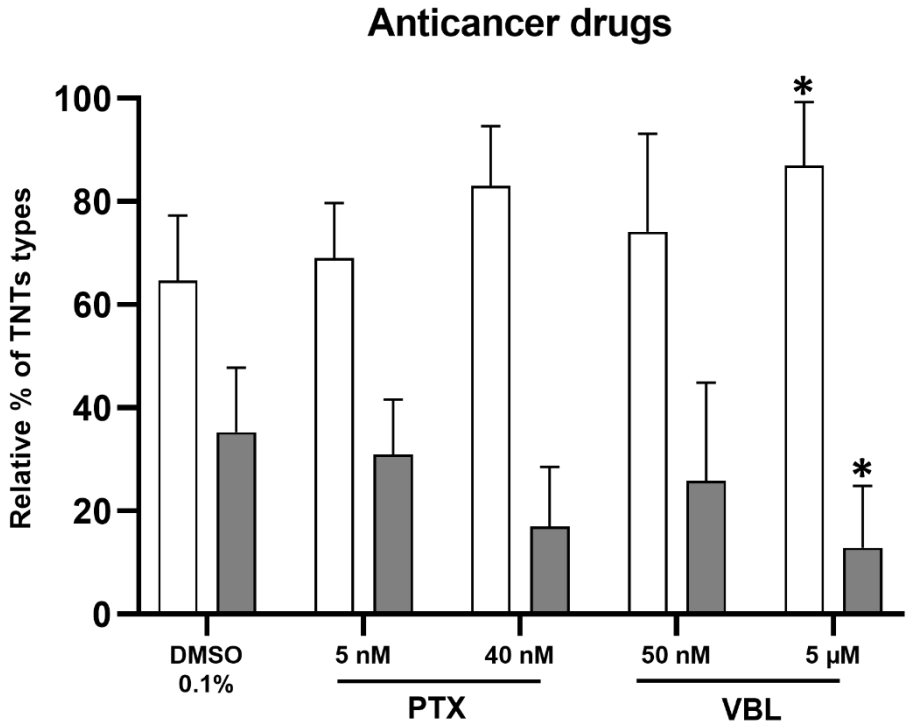



**Figure 16: Effect of PTX and VBL on the relative percentage of the two types of TNTs ("thin" and "thick), after 24 hours of incubation, under normoxia conditions.** The number of TNTs/cell in MDA-MB-231 cells was quantified manually by analyzing the phase contrast images: "Thin" TNTs (open bars) are considered those with diameters < 0.7µm, and "thick" TNTs (grey bars) those with diameters ≥ 0.7µm. The detailed method of quantification of TNTs can be found in Materials and Methods, section 3.7. Data from five independent experiments are presented as mean ± S.D. for each compound (three replicates). Statistically significant differences from the respective control: *p<0.05 (Student´s *t*-test). PTX: paclitaxel**;** VBL: vinblastine.

For the lowest concentration of vinblastine and the highest concentration of paclitaxel tested, there seems to be a tendency for a decrease in the percentage of "thick" TNTs, although no significant differences were found.

When cells were treated with the anthelmintic drugs, flubendazole and mebendazole at the tested concentrations did not influence the percentage of "thick" TNTs (**Figure 17A** and **Figure 17B**, respectively). However, when cells were treated with nocodazole, at the lowest concentrations tested (25 and 50 nM), no effect on the relative percentages of the two types of TNTs were observed. In contrast, at the highest concentrations tested (100 and 200 nM) a significant reduction of the percentage of "thick" TNTs was observed (**Figure 17C**).

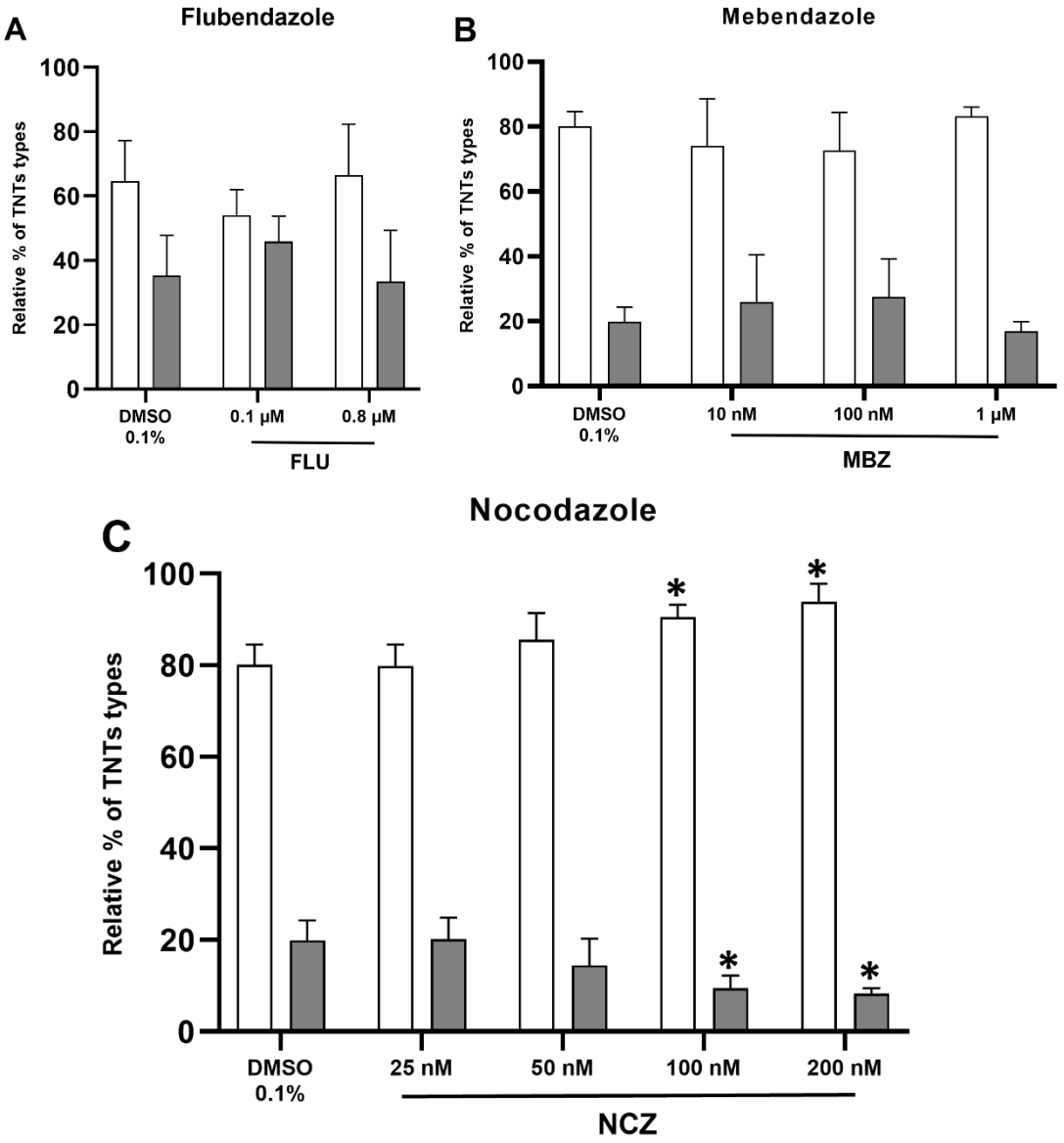



**Figure 17: Effect of anthelmintic drugs (A) FLU, (B) MBZ, and (C) NCZ on the thickness/diameter of TNTs after 24h of incubation under normoxia conditions.** The number of TNTs per cell in MDA-MB-231 cells was quantified manually by analyzing the phase contrast images: "Thin" TNTs (open bars) are considered those with diameters < 0.7µm, and "thick" TNTs (grey bars) those with diameters ≥ 0.7µm. The detailed method of quantification of TNTs can be found in the section Materials and Methods, section 3.7. Data from three independent experiments are presented as mean ± S.D. for each compound (three replicates). Statistically significant differences from the respective control: *p<0.05. (Student´s t-test). FLU: flubendazole; MBZ: mebendazole; NCZ: nocodazole.

Hypoxic conditions within tumors form oxygen gradients, increasing tumor diversity and aggressiveness[157]. Therefore, the study of the effects of the drugs mentioned above in hypoxic conditions and comparisons with the results obtained in normoxia is very pertinent. Therefore, the impact of the anticancer and anthelmintic drugs on MDA-MB-231 cell viability and their effects on TNT formation and the tickness of these structures (types of TNTs formed), similar experiments were carried out in a chemically-induced hypoxic environment. For this set of experiments, cells were incubated in culture medium containing 25 µM CoCl2, which showed to be the most adequate concentration of the chemical agent that mimics hypoxic conditions for this purpose in preliminary experiments. For details, see Materials and Methods, Section 3.3).

## 4.4. Effect on cell viability of MDA-MB-231 cells after treatment with antimitotic drugs under hypoxia conditions

**Figure 18A** illustrates the effect of antimitotic drugs currently used in multiple anticancer therapies, specifically paclitaxel and vinblastine, in increasing concentrations on the cell viability of MDA-MB-231 cells. A decrease in cell viability was observed for paclitaxel at the highest concentration (40 nM), whereas the lowest concentration of this drug caused only a small decrease in cell viability. The two concentrations of vinblastine studied elicited a decrease in cell viability. Regarding anthelmintic drugs, flubendazole, at the highest concentration tested, caused a decrease in cell viability, as seen in **Figure 18B**.

These results are similar to those obtained for the same drug concentrations under normoxic conditions (**Figure 12**).

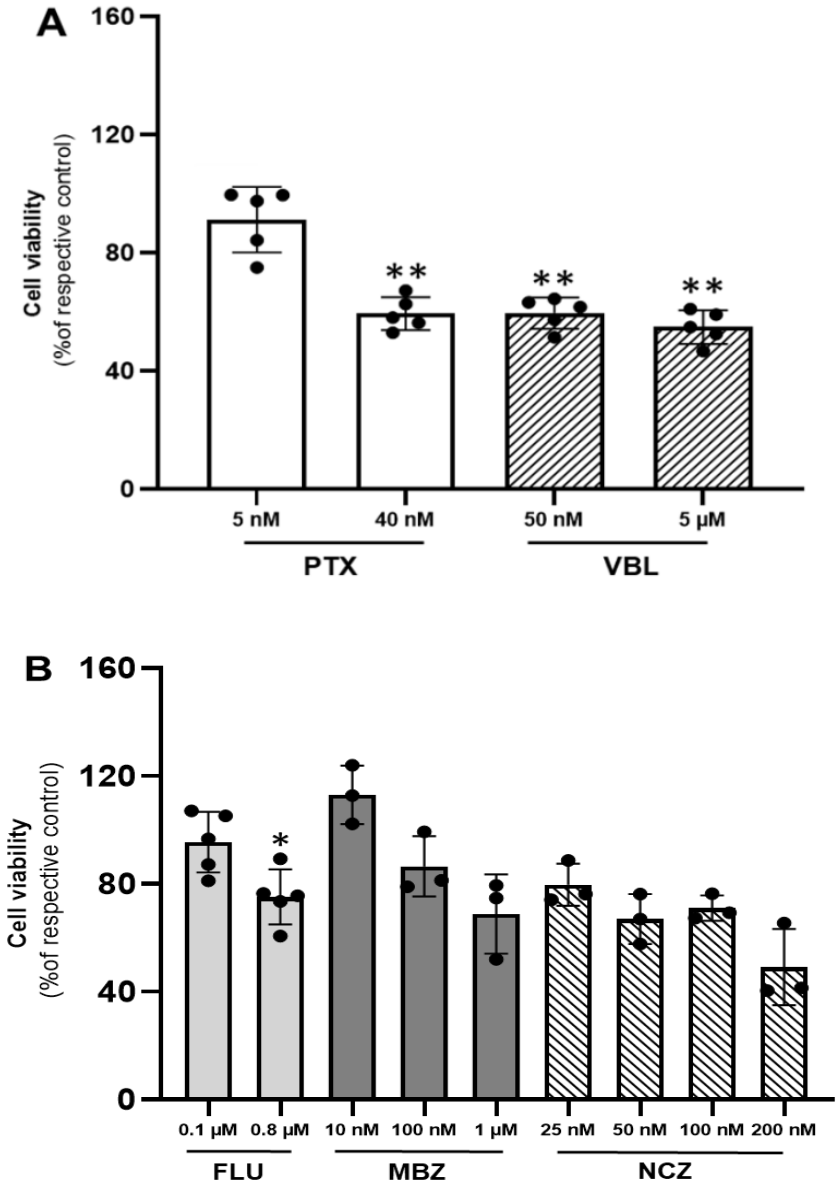



**Figure 18: Effect of drugs used in therapy as anticancer (A) and anthelmintic drugs (B) on MDA-MB-231 cell viability, after 24 h incubation, under hypoxia conditions.** MDA-MB-231 cells were seeded for adhesion and then treated with the indicated drugs at increasing concentration for 24 hours in conditions of chemically induced hypoxia (25 µM CoCl2). Cell viability was evaluated using the PrestoBlue™ reagent assay. The results are expressed as a percentage of the respective control (cells treated with DMSO 0.1%). Data from three to five independent experiments are presented as mean ± S.D. for each compound (three replicates). Statistically significant differences from the respective control: *p<0.01; **p<0.001. (Student´s *t*-test). PTX**:** paclitaxel**;** VBL: vinblastine**;** FLU: flubendazole**;** MBZ: mebendazole**;** NCZ**:** nocodazole

## 4.5. Effect of antimitotic anticancer drugs under hypoxic conditions on TNTs formation

When exposed to different concentrations of paclitaxel in hypoxic conditions (grey boxes), the results reflect those observed in normoxic conditions (white bars), **Figure 19**. Specifically, for the highest concentration of the drug, in both conditions, a decrease in the number of TNTs/cell was observed. It seems that the lowest paclitaxel concentration there is also a decrease in the number of TNTs/cell but it is not statistically significant.

Moreover, no differences were observed between similar paclitaxel treatments carried out in normoxic or hypoxic conditions.

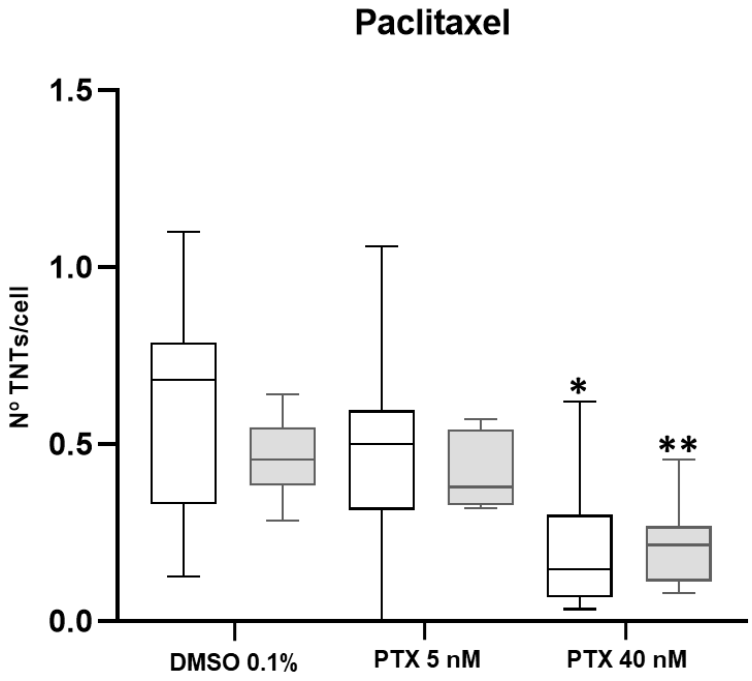



**Figure 19: Effect of PTX treatment (24h) on the number of TNTs/cell in MDA-MB-231 cells, under normoxia (white bars) and hypoxia (grey bars) conditions.** The quantification of the number of TNTs was done manually through the analysis of phase contrast images captured by Lionheart FX. The detailed method of quantification of TNTs can be found in Materials and Methods, section 3.7. Data from five independent experiments are expressed as median and interquartile ranges of three wells for each compound (three replicates). Normoxic conditions: white boxes; hypoxic conditions: grey boxes. Statistically significant differences from control: *p<0.01; **p<0.0001; (Student´s *t*-test). PTX: paclitaxel.

Concerning cell treatments with vinblastine, in hypoxic conditions (**Figure 20**), both concentrations of this drug tested decreased the number of TNTs/cell compared to the control [DMSO 0.1% ($CoCl_2$)], similarly to that observed in normoxic conditions. Moreover, no other significant differences were found when similar treatments (drug and concentration), carried out in normoxic and hypoxic conditions.

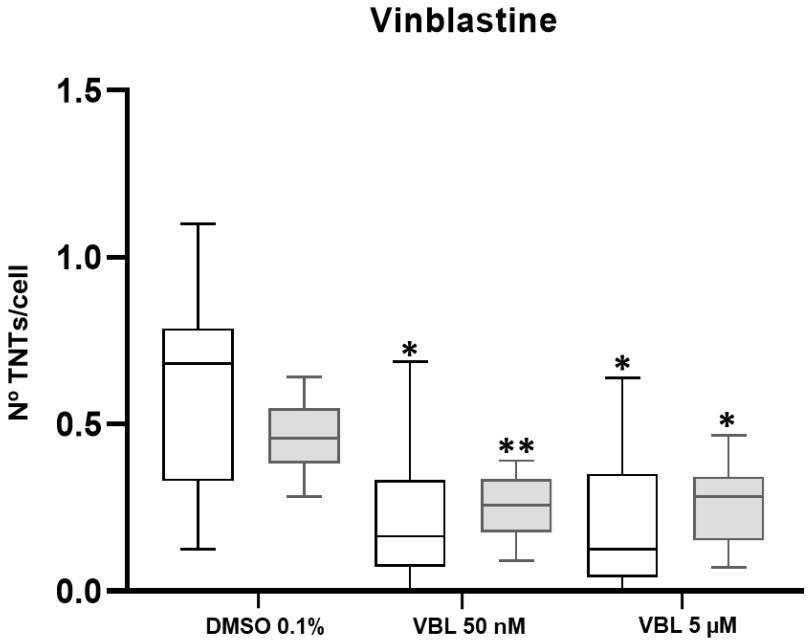



**Figure 20: Effect of MDA-MB-231 cells treatment with vinblastine for 24 hours on the number of TNTs/cell, under normoxic and hypoxic conditions.** The quantification of the number of TNTs was done manually through the analysis of phase contrast images captured by Lionheart FX. The TNT quantification method is detailed in Materials and Methods, section 3.7. Data from five independent experiments are expressed as median and interquartile ranges of three wells for each compound (three replicates). Normoxic conditions: white boxes; hypoxic conditions: grey boxes. Statistically significant differences from control: *p<0.01; **p<0.0001; (Student´s *t*-test). VBL: vinblastine.

## 4.6. Effect of anthelmintic drugs under hypoxic conditions on TNTs formation

This section presents the results obtained for the cell treatment with the anthelmintic drugs (flubendazole, mebendazole, and nocodazole) on the formation of TNTs under hypoxic conditions.

Presented below are the results of flubendazole regarding its effect on the number of TNTs/cell, under hypoxic conditions, **Figure 21** (**grey bars**). Of the two concentrations tested, we could observe what appears to be a decrease in the number of TNTs/cell for the highest concentration tested (0.8 µM). However, as in normoxic conditions, there were no significant differences compared to the control. When comparing the controls and each concentration of vinblastine under normoxic and hypoxic conditions, we also could not find statistically significant differences.

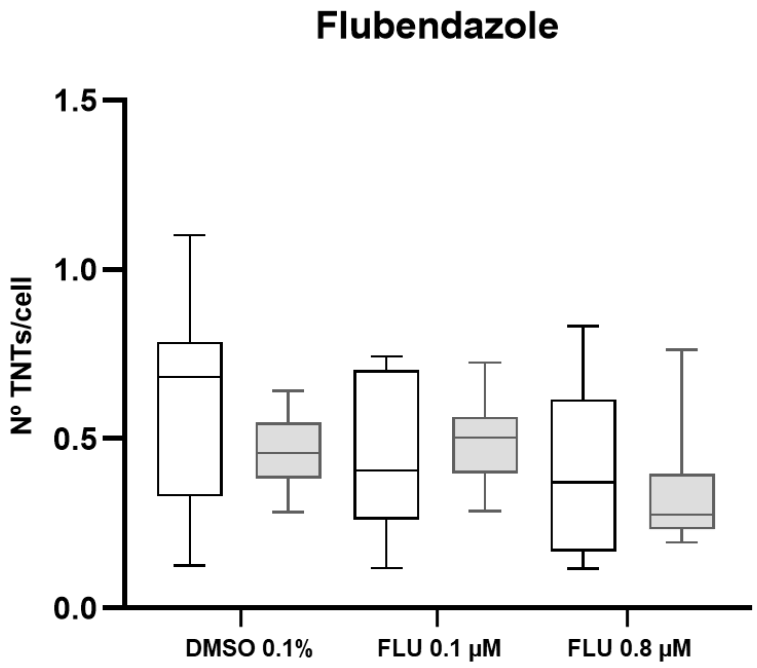



**Figure 21: Effect of FLU treatment (24h) on the number of TNTs/cell in MDA-MB-231 cells, under normoxia (white bars) and hypoxia (grey bars) conditions.** The quantification of the number of TNTs was done manually through the analysis of phase contrast images captured by Lionheart FX. The detailed method of quantification of TNTs can be found in Materials and Methods, section 3.7. Data from five independent experiments are expressed as median and interquartile ranges of three wells for each compound (three replicates). Normoxic conditions: white boxes; hypoxic conditions: grey boxes. Statistically significant differences from control There are no significant differences between the tested concentrations with the control. (Student´s *t*-test). FLU: flubendazole.

In hypoxic conditions, **Figure 22**, the drug mebendazole at concentrations of 100 nM and 1μM presents a tendency to decrease the number of TNTs/cell, and at 10 nM, there seems to occur as light increases in the number of TNTs/cell. Once more, no significant differences were found between treatments and controls (cells incubated DMSO 0.1%, in the presence of 25 μM ($CoCl_2$).

In contrast to the results observed with the compounds examined above, a significant difference in the number of TNTs/cell was observed when comparing cell treatments with 100 nM mebendazole in normoxic and hypoxic conditions. No significant effects were observed for the other concentrations.

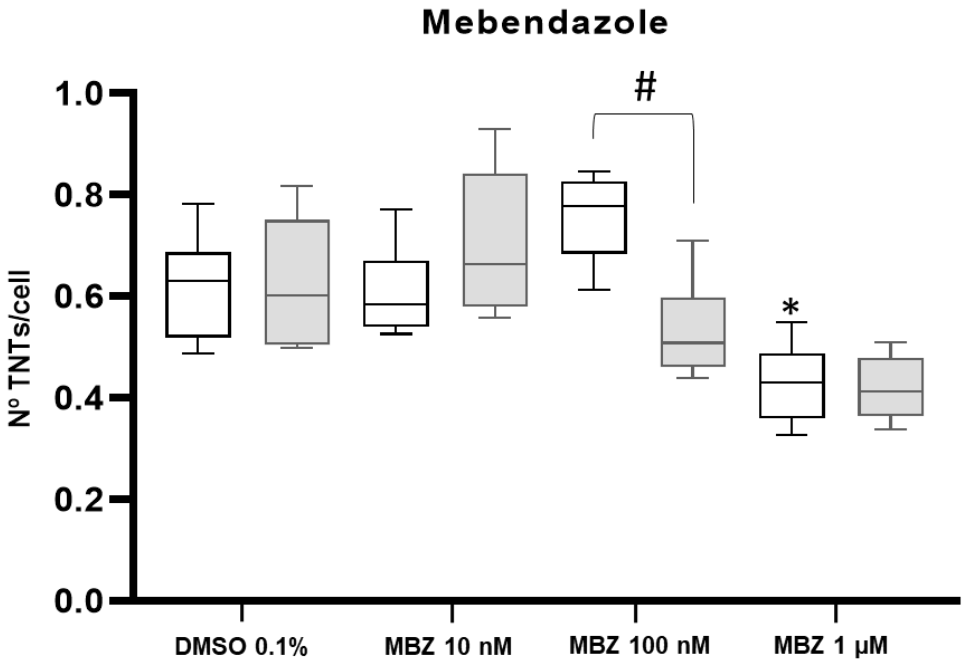



**Figure 22: Effect of MBZ treatment (24h) on the number of TNTs/cell in MDA-MB-231 cells, under normoxia (white bars) and hypoxia (grey bars) conditions.** The quantification of the number of TNTs was done manually through the analysis of phase contrast images captured by Lionheart FX. The detailed method of quantification of TNTs can be found in Materials and Methods, section 3.7. Data from three independent experiments are expressed as median and interquartile ranges of three wells for each compound (three replicates). Normoxic conditions: white boxes; hypoxic conditions: grey boxes. Statistically significant differences from control: *p<0.05; #p<0.01. (Student´s t-test). MBZ: mebendazole.

Concerning nocodazole (**Figure 23**), a decrease in the number of TNTs/cell for three of the concentrations tested (50 nM, 100 nM, and 200 nM), similar to that observed in experiments carried out under normoxic conditions (white bars). For the lowest nocodazole concentration studied (25 nM), there is a tendency to decrease in the number of TNTs/cell, which was not statistically significant. There were no significant differences between the controls and treatments carried out in normoxic or hypoxic conditions.

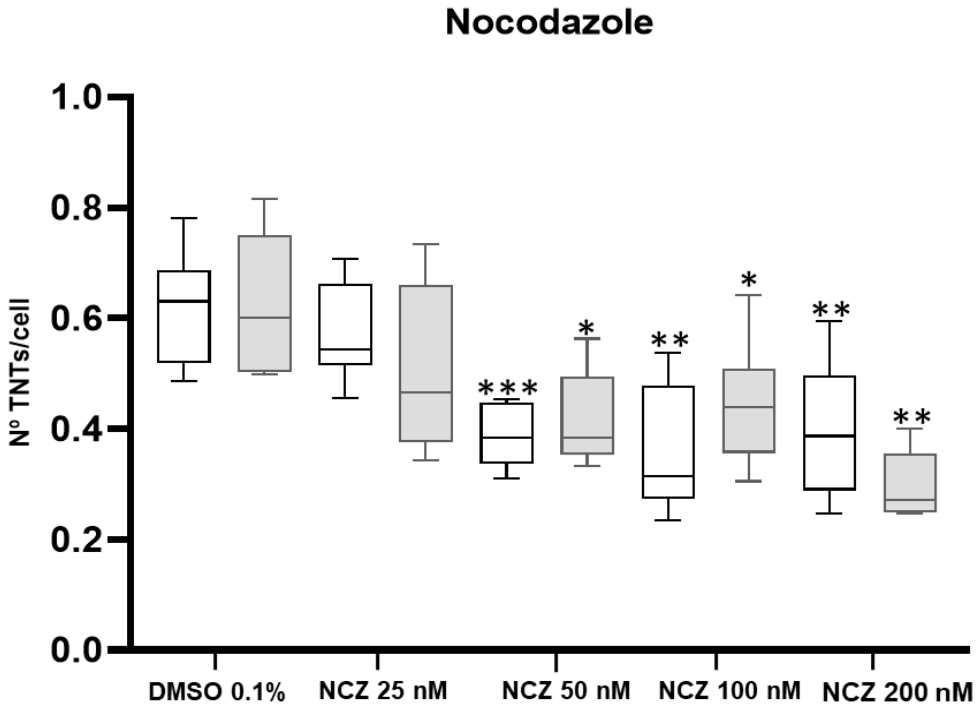



**Figure 23: Effect of NCZ treatment (24h) on the number of TNTs/cell in MDA-MB-231 cells, under normoxia (white bars) and hypoxia (grey bars) conditions.** The quantification of the number of TNTs was done manually through the analysis of phase contrast images captured by Lionheart FX. The detailed method of quantification of TNTs can be found in Materials and Methods, section 3.7. Data from three independent experiments are expressed as median and interquartile ranges of three wells for each compound (three replicates). Normoxic conditions: white boxes; hypoxic conditions: grey boxes. Statistically significant differences from control: *p<0.05; **p<0.01; ***p<0.001 (Student´s *t*-test). NCZ: nocodazole.

## 4.7. Influence of antimitotic and anthelmintic drugs on the diameter of TNTs under hypoxia conditions

In this final aspect, a comparison is conducted for each drug under both conditions to assess their impact on the size of TNTs.

Only the highest concentration of paclitaxel studied (40 nM; **Figure 24A**) was able to decrease the percentage of "thick" TNTs comparatively to the control group. A similar effect was observed for the highest concentration of vinblastine, as illustrated in **Figure 24B**. In the case of the lower concentrations of the anti-mitotic drugs, a tendency for a decrease in the percentage of "thick" TNTs in hypoxic conditions was observed, although no significant differences occurred.

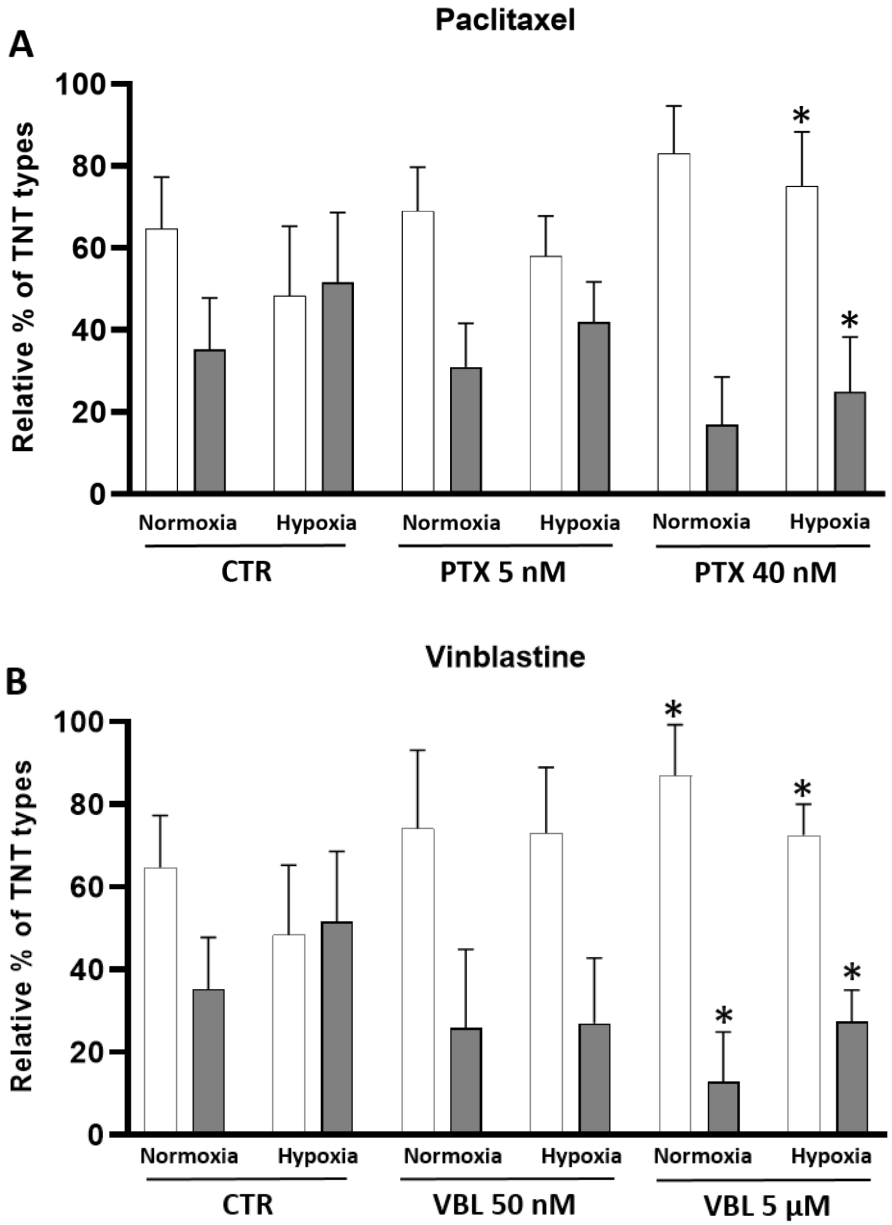
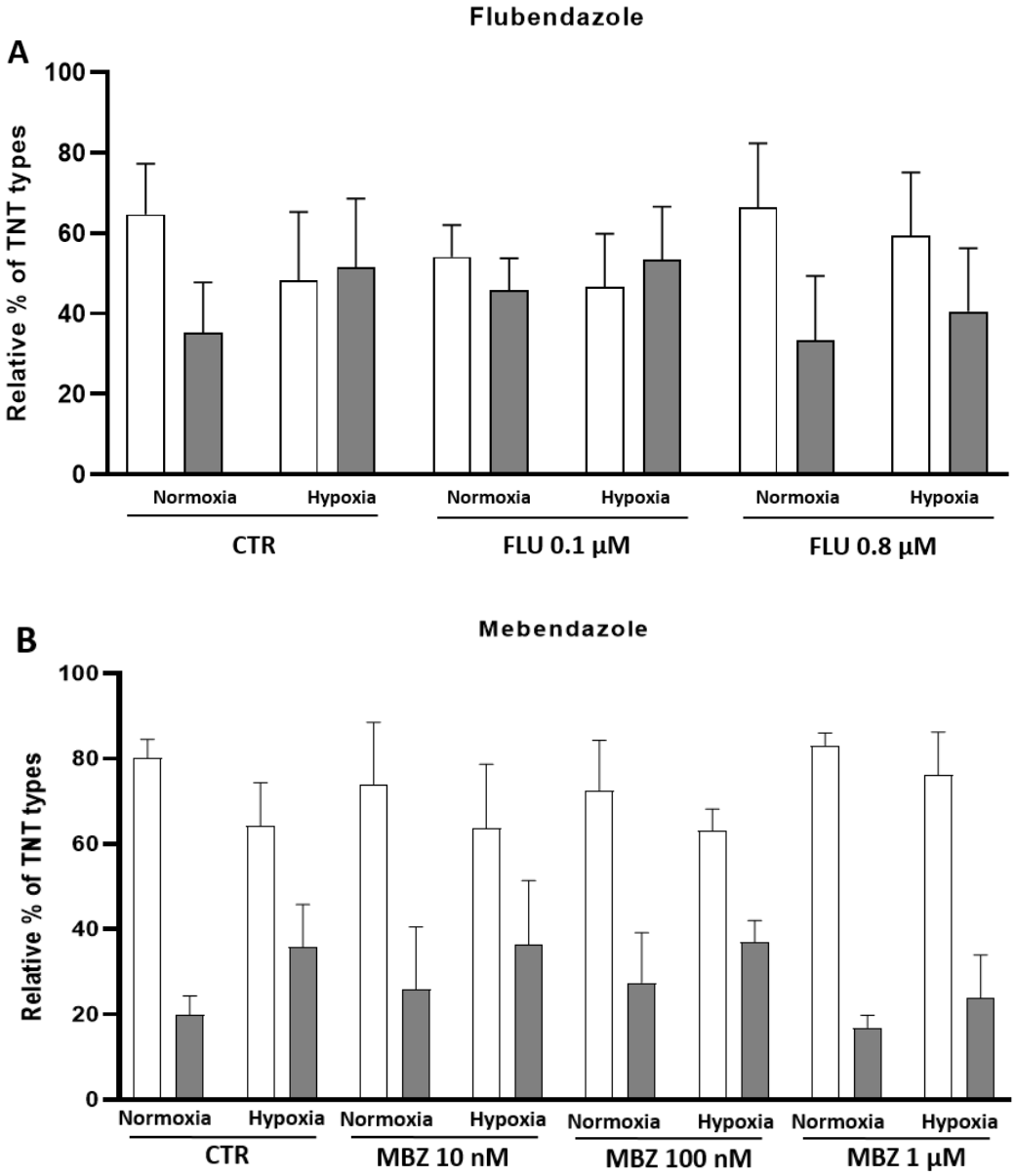



**Figure 24: Effect of PTX and VBL on the size of TNTs after 24h of incubation under normoxia and hypoxia conditions.** The number of TNTs per cell in MDA-MB-231 cells was quantified manually by analyzing the phase contrast images: "Thin" TNTs (grey bars) are considered those with diameters < 0.7µm, and "thick" TNTs (white bars) those with diameters ≥ 0.7µm. The detailed method of quantification of TNTs can be found in the section Materials and Methods, section 3.7. Data from three independent experiments are presented as mean ± S.D. for each compound (three replicates). Statistically significant differences from the respective control: *p<0.05; (Student´s t-test). PTX: paclitaxel; VBL: vinblastine.

Among the anthelmintics evaluated for the drug flubendazole (**Figure 25A**) at the highest concentration, under hypoxic conditions, there appears to be a slight decrease in the percentage of "thick" TNTs compared to the control, but no significant differences occurred. Concerning mebendazole (**Figure 25B**) at concentrations of 100 nM and 1 µM, similarly, a tendency for a decrease percentage of "thick" TNTs seems to occur although no significant differences were observed. A similar pattern was observed for all concentrations of nocodazole tested (**Figure 25C**)., there appears to be a decrease in the percentage of "thick" TNTs for the concentrations tested under the same conditions, but there are no significant differences.



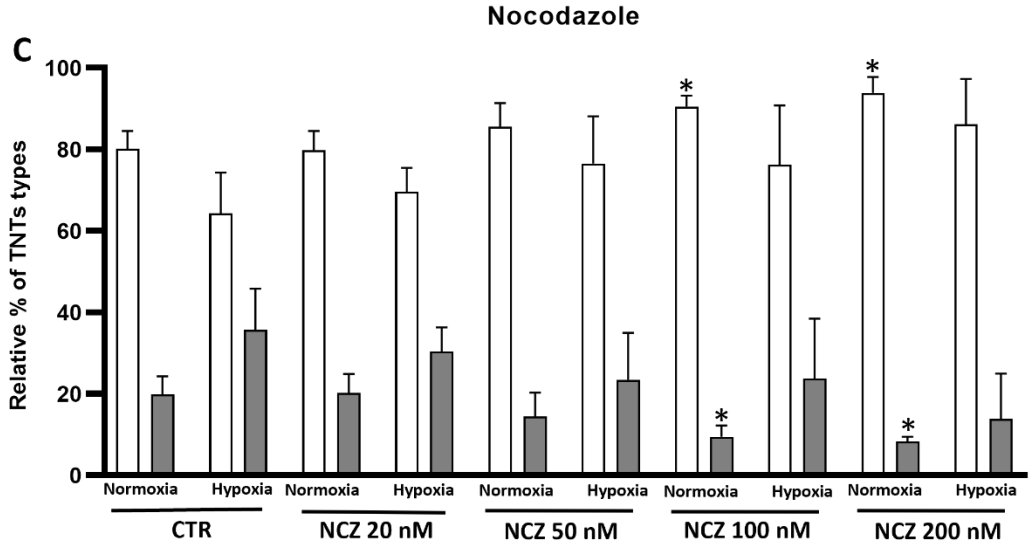
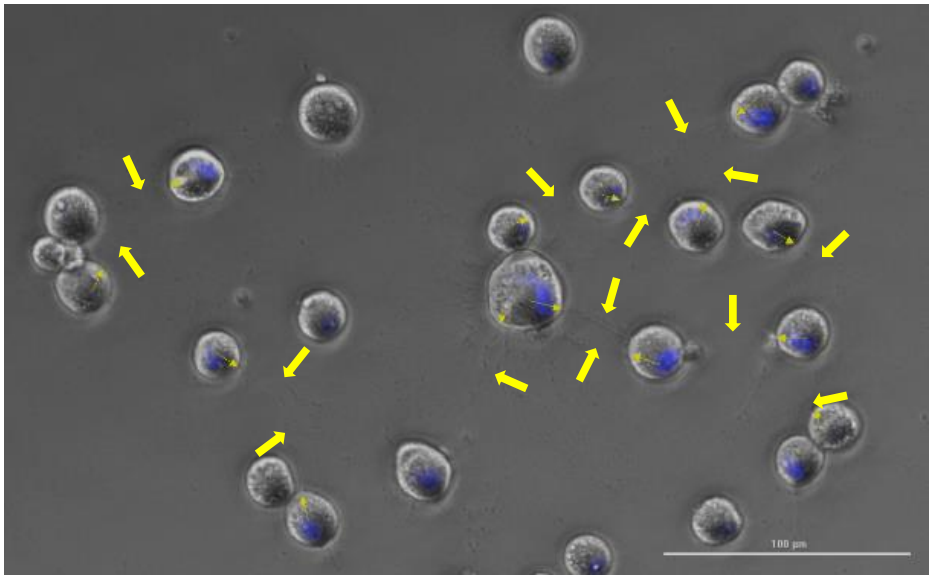



**Figure 25: Effect of anthelmintic drugs (A) FLU, (B) MBZ, and (C) NCZ on the size of TNTs after 24h of incubation under normoxia and hypoxia conditions.** The number of TNTs per cell in MDA-MB-231 cells was quantified manually by analyzing the phase contrast images: "Thin" TNTs (grey bars) are considered those with diameters < 0.7µm, and "thick" TNTs (white bars) those with diameters ≥ 0.7µm. The detailed method of quantification of TNTs can be found in the section Materials and Methods, section 3.7. Data from five independent experiments are presented as mean ± S.D. for each compound (three replicates). Statistically significant differences from the respective control: *p<0.05; (Student's t-test). FLU: flubendazole; NCZ: nocodazole; MBZ: mebendazole.

## 4.8. Quantification of number of TNTs/cell using images of cells stained with Flash Phalloidin™ green 488

For the F-actin staining experiments, after capturing images with Lionheart FX in phase contrast (**Figure 26A**), cells were fixed as described in Materials and Methods, section 3.6, stained with Flash marker Phalloidin™ green 488 and new images were captured using the GFP channel of the automated inverted microscope Lionheart FX (**Figure 26B**). The quantification of TNTs/cell was subsequently conducted, using both phase contrast microscope images and fluorescence data, and the results were subsequently compared. These comparative experiments revealed a lower number of TNTs (or even total absence of these structures) using the F-actin fluorescence images. As described in the literature, TNTs are sensitive to fixation processes[168], which explain these results. The microphotographs presented in **Figure 26** are representative and show images of the same region of the same well obtained using phase contrast and GFP fluorescence modes (**Figure 26A and B**, respectively).

A



B

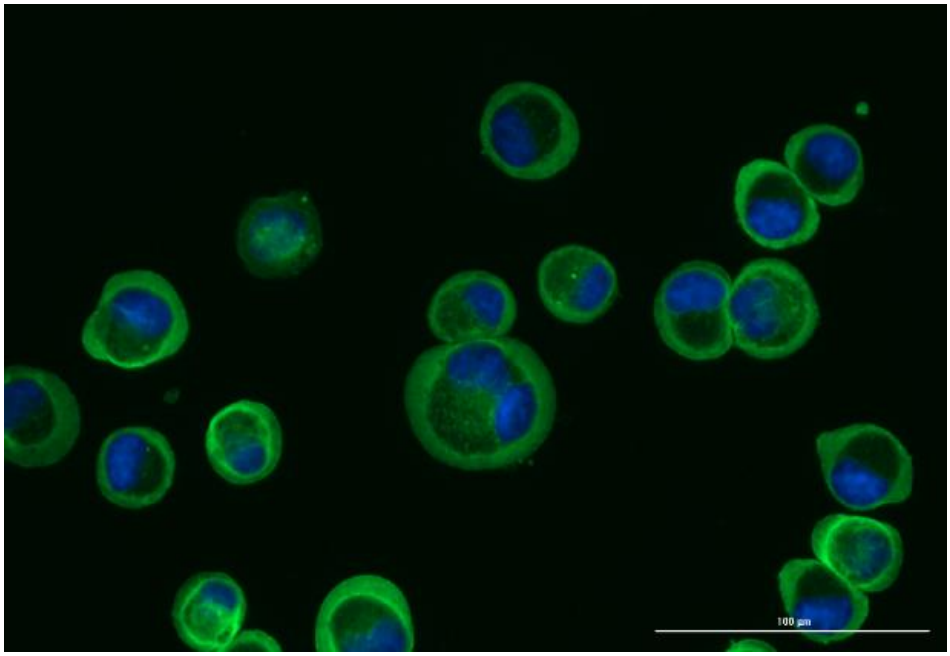


**Figure 26**: **Microphotographs of cells for the quantification of TNTs using representative microphotographs acquired in phase contrast (A) and fluorescence mode (GFP channel; Flash Phalloidin™ green 488) (B).** The number of TNTs (yellow arrows) per cell in MDA-MB-231 cells was quantified manually by analyzing images captured by the Lionheart FX, as described in Materials and Methods, section 3.6. Nuclei (blue fluorescence) were labelled with Hoechst 33342. Scale bar: 100 µm.

**Table 3** shows representative results of the comparison of the quantification of the number of TNTs/cell, which demonstrate the loss of TNTs after fixation of the cells with F-actin.

**Table 3**: Number of TNTs/cell quantified in microphotographs of the same region of individual wells acquired in fluorescence or phase contrast images

| Experiment | Well | Flash Phalloidin™ green 488 | Phase contrast |
|---|---|---|---|
| **Experiment 1** | Control | 0.031 | 0.740 |
| | Treatment 1 | 0.061 | 0,527 |
| | Treatment 2 | 0 | 0.097 |
| | Treatment 3 | 0 | 0.330 |
| | Treatment 4 | 0.018 | 0.456 |
| **Experiment 2** | Control | 0.025 | 0.691 |
| | Treatment 1 | 0.062 | 0.407 |
| | Treatment 2 | 0.010 | 0.202 |
| | Treatment 3 | 0.073 | 0.391 |
| | Treatment 4 | 0 | 0.339 |

Data presented are number of TNTs/cell quantified as described in Materials and Methods, section 3.7, using images of the same region of each plate well (with or without drugs) acquired in fluorescence and phase contrast modes by the automated microscope Lionheart FX.

# V. Chapter: Discussion

## 5. Discussion

The present study reveals the capacity of anticancer drugs that target microtubules to interfere with TNTs formation.

Despite the importance of TNTs, they are difficult to quantify, and it is, therefore, difficult to evaluate the effect of drugs on their formation. The methodological difficulties justify the lack of knowledge about the pharmacological effects on TNTs. A prime illustration of this is the limited literature addressing the impact of anticancer drugs that disrupt the mitotic spindle on TNT formation. Remarkably, only one study within the literature delves into evaluating an anticancer drug's effect on TNT formation[75]. In this study, the authors show the influence of paclitaxel on TNT formation, ultimately concluding that this drug reduces the formation of these structures in prostate cancer cells[75].

The present study assessed the influence of antimitotic drugs, paclitaxel and vinblastine, on cell viability and TNTs formation in breast cancer cells, specifically the MDA-MB-231 cell line. The findings reveal that upon exposure to these conventional anticancer agents, caused a reduction in the number of formed TNTs in MDA-MB-231 cells. This original observation was accompanied by a decrease in cell viability, as expected.

TNTs may be targeted by drugs other than the oncological antimitotic drugs. Drug repurposing is a strategy that is becoming increasingly popular to speed up the development of new drugs. This is because some stages of the process, such as assessing the safety and efficacy of drugs, have already been approved, which makes it possible to access drugs at a lower cost[169].

As cancer is one of the most significant global health threats, the challenge resides in applying the drug repurposing approach to search for drugs with anti-cancer potential[150], as is the case with the anthelmintics discussed in this work.

Anthelmintics, especially benzimidazole derivatives, exhibit anticancer properties by inhibiting cell proliferation and inducing apoptosis, as documented in the literature[124, 150, 170, 171]. Given the analogous mechanism of action shared by anthelmintic drugs, specifically benzimidazole derivatives, with anti-cancer drugs, it is possible that they could influence the formation of TNTs similarly.

To date, no studies within the anthelmintic drug class have been conducted to investigate their impact on TNTs formation, rendering our research groundbreaking and pioneering.

The present study also investigated the effects on the different types of TNTs. TNTs can be distinguished into "thin" TNTs (made up only of actin) and "thick" TNTs depending on their

constitution; they serve as direct communication pathways between cells, allowing the transfer of molecules essential for intercellular communication[63].

The present study reveals a capacity of the anthelmintic drugs to inhibit TNTs formation. This effect occurs only in the thicker TNTs, the ones that are composed of actin and tubulin. No significant effects were observed in the thin TNTs, the ones that are mainly composed of actin polymers. Among the anthelmintic drugs tested, nocodazole was the most potent drugs while flubendazole was shown to be the least potent drug, apparently. In general, these effects on TNTs were accompanied by a tendence to decrease cell viability what may indicate that TNTs may play a relevant role in the survival of metastatic breast cancer cells.

The effects of anthelmintic drugs on TNTs may not be linear. It was observed that mebendazole at the intermediate concentration tested (100 nM) increased TNTs formation and caused a decrease with the other concentrations tested. Additional experiments are warranted to gain a fully understanding of how anthelmintic drugs influence TNTs the dynamics.

It is known that in some tumors regions, cells are under hypoxic conditions and this kind of stress may induce a metabolic reprograming that may cause drug resistance[172, 173]. Resistance to chemotherapeutic drugs remains a significant challenge in oncology, leading to a rise in cancer-related mortality, particularly in cancer types with high prevalence, such as breast cancer. One illustrative aspect of a pivotal mechanism underlying the arrangement and diversity of tumor cells, potentially elucidating the emergence of chemoresistance, pertains to intercellular communication. The importance of TNTs in cancer, as well as in various disease models, presents a new and important area of exploration. This research is fundamental to understanding the mechanisms that govern long-range cellular communication in tumors' complex and diverse microenvironments.

In the present study the possibility that effects of antimitotic drugs and anthelmintics on TNTs may be different on tumor cells with or without hypoxia-induced metabolic reprograming, a parallel set of experiments was carried out under conditions that mimic hypoxia. In hypoxic environments, tumor cells tend to develop resistance to various treatments.

In this work, cobalt chloride ($CoCl_2$) was employed to simulate hypoxia. This compound is well-documented in the literature as a hypoxia-mimicking agent. It is known to induce the expression of the HIF-1α protein and modulate the expression of genes associated with hypoxia, particularly those involved in angiogenesis and apoptosis, within the cell line used in our study[174, 175].

No significant differences were found between the outcomes obtained under hypoxic and normoxic conditions. Across all drugs examined, there was a consistent trend of diminishing TNTs formation as observed under normoxic conditions. These findings suggest that these drugs will be equally effective on TNTs of tumor cells irrespectively of the hypoxic influence on their metabolism. Such property is highly relevant as it is a condition of not contributing to the development of resistance to chemotherapy treatment.

With this approach, it was possible to reveal that TNT formation may be pharmacologically modulated. To our knowledge, this is the first study showing that TNTs may also be a target to the antimitotic drugs and also to some repurposed drugs, as it is the case of benzimidazole anthelmintic drugs. The present work should be seen as a first approach to explore the pharmacology of TNTs. Understanding the molecular dynamics of TNTs may offer the opportunity to increase the pharmacological armamentarium with drugs able to selectively modulate their effects. As shown, anthelmintic drugs have the potential to be further explored and nocodazole appears to be the most promising drug.

Still, the correlation between molecular structure and biological activity should also be taken into consideration to explore the discovery of new ligands with higher potency and selectivity. The benzimidazole structure present in all compounds tested may give an indication of some structure-activity requirements[176, 177]. Moreover, variations in the functional groups in the molecular structures can also influence the binding region of the drug, the binding affinity to the targets, and, consequently, the efficacy of these drugs. The need for further research in this field is evident because the structure-activity relationship (SAR) extends beyond chemical composition to include selectivity for specific molecular targets in cancer cells.

# VI. Chapter: Conclusion

# 6. Conclusion

A method that allowed to quantify drug effects on TNTs formation was successfully implemented and validated in the present study.

Antimitotic drugs were shown to influence TNTs dynamics and cancer cell proliferation, indicating that TNTs may have a role in cancer progression and be a potential innovative pharmacological target in oncology.

Benzimidazole anthelmintic drugs were also shown to influence TNT dynamics, indicating that repurposed drugs may also be used to modulate this pathway, at least in metastatic breast cancer cells.

The differences in activity of the benzimidazole drugs support the need for deeper structure-activity studies to study the molecular requirements to obtain more active and selective TNT drugs.

Differences in tumor cell response and resistance to chemotherapy may arise from the different metabolism induced by hypoxia within the tumor. Effects of these drugs on cancer cell do not seems to be influenced by differences on the metabolic program induced by hypoxia and, thus, the less prone to promote resistance.

The results obtained in this study led us to the conclusion that antimitotic drugs, which are recognized for their interference with cancer cells, specifically those targeting tubulin, and anthelminthic drugs, which hold promise as repurposing drugs in oncology, possess the capacity to interfere with the formation TNTs.

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