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Apoptosis and paraptosis, involving endoplasmic reticulum stress, autophagy and mitochondrial dysfunction, are induced by δ-tocotrienol in prostate cancer cells

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INTRODUCTION

1. PROSTATE CANCER

1.1. Anatomy and physiology of the prostate

The prostate is a gland of the male reproductive system. It does not have a proper capsule, but it is surrounded by an integral fibromuscular band. It is located in front of the rectum and just below the bladder. It is about the size of a chestnut and conical in shape, consisting of a base, an apex, an anterior, a posterior and two lateral surfaces. The base is directed upward near the inferior surface of the bladder. The apex is directed downward and is in contact with the superior fascia of the urogenital diaphragm [1,2].

The prostate weighs about 20 g, and it is 3 cm long, 4 cm wide and 2 cm thick [1,2].

Three histological cell types of cells can be found in the gland: glandular cells, myoepithelial cells and subepithelial interstitial cells [3].

The prostate can be sub-divided in two ways, by zone or by lobe [1,2].

The “zone” classification is commonly adopted in pathology (**fig. 1**). On the basis of this classification, the prostate is composed of:

- The peripheral zone: it is the largest area of the prostate, representing the 70% of the entire gland. It is the sub-capsular portion of the posterior side of the prostate, surrounding the distal urethra. It can be palpated through the rectum during the digital rectal exam. Almost 70-80% of prostate cancers (PCas) originate in this zone.
- The transition zone: it surrounds the prostatic urethra. It represents about 5-10% of the prostate volume but dramatically grows throughout life, sometimes leading to benign prostatic hyperplasia.
- The central zone: it lies behind the transition zone and surrounds the ejaculatory ducts, representing 25% of the prostate volume. Only 2.5% of cancers start in this region but they tend to be more aggressive and metastatic.
- The anterior fibromuscular stroma: this zone, wich surrounds the apex of the prostate, is composed of muscle fibers and fibrous connective tissue and does

not contain any glandular components. PCa is rarely found in this part of the prostate.

The “lobe” classification is generally used in anatomy. The prostate is divided into five lobes:

- The posterior lobe roughly corresponds to the peripheral zone.
- The anterior lobe roughly corresponds to part of the transition zone.
- The median lobe roughly corresponds to part of the central zone.
- The lateral lobes (right and left lobes) form the main mass of the gland and are located posteriorly. They are separated by the prostatic urethra.

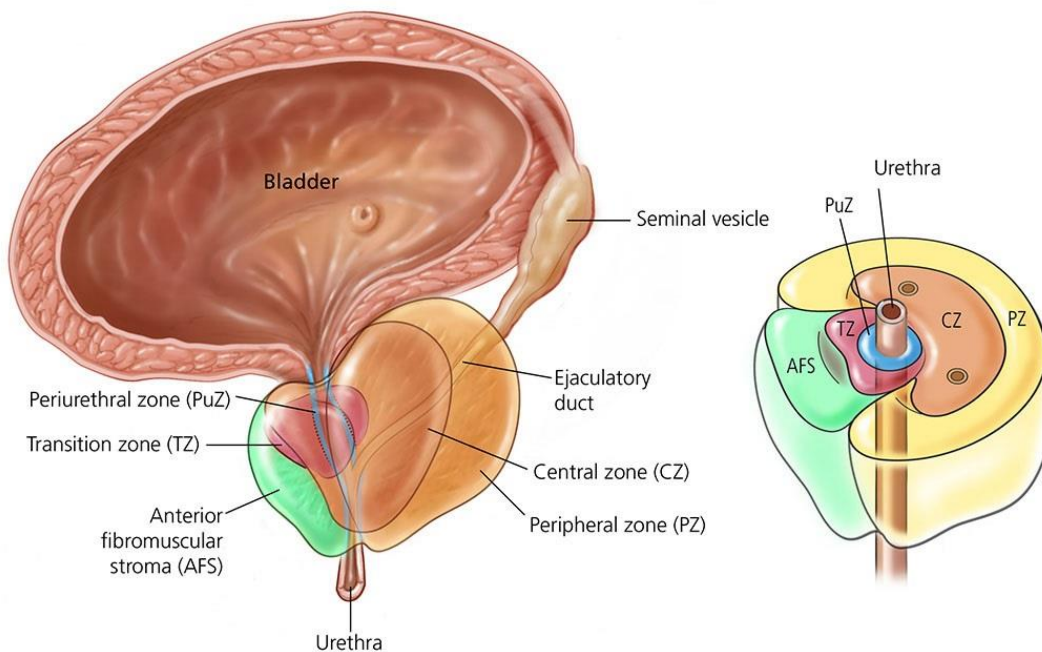

***Fig. 1**. Prostate anatomy.*

The main function of the prostate is to secret the prostatic fluid through the ducts and into the urethra, where it mixes with sperm and is ejaculated as semen. Prostatic fluid forms about 20% of semen volume and contains spermine, spermidine, prostaglandins, zinc, citric acid, immunoglobulins, phosphatases and proteases [4].

The prostatic urethra originates from the pelvic portion of the urogenital sinus. Endodermal outgrowths develop from the prostatic urethra and grow into the

surrounding mesenchyme. The endodermal cells differentiate into the glandular epithelium, while the dense stroma and the smooth muscle of the prostate differentiates from the associated mesenchyme. The prostate glands represent the modified wall of the proximal part of the male urethra and develop by the ninth week of embryonic life. Fusion of mesenchyme, urethra and Wolffian ducts gives rise to the adult prostate gland [3,5].

Notably, androgens, particularly testosterone, are known to be essential for prostate development and function, as well as for proliferation and survival of cells within the gland [3,5].

1.2. Epidemiology of prostate cancer

The global burden of PCa is substantial, ranking among the top five cancers for both incidence and mortality [6]. In particular, PCa is characterized by striking geographical variation in both incidence and mortality rates. In this context, the analysis of PCa incidence and mortality across populations may clarify the role of individual risk factors in the epidemiology of this disease.

1.2.1. Incidence

Globally, PCa is the most commonly diagnosed cancer in men, being particularly common in developed countries [7].

As mentioned above, PCa incidence is remarkable for its substantial global variation. There is a 40-fold difference in age-adjusted incidence rates between men with the highest (African-American men in the United States) and lowest (Asian men living in their native countries) incidence [8]. In part, this global variation can be related to differences in the prostate-specific antigen (PSA) screening frequency. Indeed, in the last forty years, age-adjusted incidence rates have generally increased across the world, a trend that has been accompanied by a crescent use of PSA testing in certain regions, including the United States, Europe and Australia. The emergence of PSA testing has also led to an increase in early diagnosis, with a higher number of men diagnosed with localized disease [9]; an increase in overdiagnosis (*i.e.*, the diagnosis of tumors that would not

have cause symptoms or death in the absence of screening) has also been observed [10,11]. However, incidence rates have also increased in regions where PSA screening has not yet been widely used, such as in Japan and some other Asian and Eastern European countries [12]. The trend in these regions suggests that environmental or lifestyle factors, as discussed later in this chapter, may also influence PCa incidence.

1.2.2. Mortality

PCa is the fifth most common cause of cancer death worldwide [7]. The highest PCa mortality rates are among populations in Middle and Southern Africa and in the Caribbean. On the contrary, the lowest PCa mortality rates are observed in Asia, particularly in Eastern and South-Central Asia. Interestingly, PCa mortality has been significantly reduced across various Westernized countries. The reasons underlying this decrease are unclear. However, the earlier identification of PCa through PSA screening and its subsequent earlier treatment could contribute to this reduction [13]. Indeed, it should be noted that some countries with low or no screening (*e.g.* Africa) are experiencing increased PCa mortality.

Considerable variability in the ratio of PCa incidence/mortality has been highlighted, with the highest ratio in North America (10:1), lower in Australia (2:1) and almost equal in some Caribbean and African countries (1.2:1). Again, these differences may be partially explained by a larger number of slow-growing tumors diagnosed in countries using PSA screening [14,15] and, conversely, by a later cancer presentation in countries with lower diagnostic intensity.

The high prevalence of PCa also reflects the magnitude of burden attributable to this disease. In fact, PCa has the highest 5-year prevalence of any cancer type, accounting for 25% of all prevalent cancers [6]. Importantly, more than four million men are PCa survivors living with a cancer diagnosis around the world [16]. This has important implications for the allocation of resources for men who are undergoing treatment or surveillance for this disease.

As shown, numerous characteristics of PCa epidemiology can be gleaned from the analysis of incidence and mortality rates across countries and over time. The evidence for specific risk factors associated with PCa will be discussed in the following paragraph.

1.2.3. Risk factors

Established risk factors for total PCa incidence include older age, African-American race and positive PCa family history. In addition, emerging evidence of genetic predisposition to PCa has been provided by genome-wide association studies (GWAS). In different ethnic groups, almost 200 genetic risk loci have been found [17,18]. Furthermore, it has been recently proposed that taller height can increase the risk of total PCa [19]. Although these factors are not modifiable, they are illustrative of the potential mechanisms involved in prostate tumorigenesis and could be used to stratify patients according to the risk of developing the disease.

Age is strongly associated with risk of total PCa. PCa is rare among men under 40 but it dramatically increases in men over 55, following a similar trend as other epithelial cancers. This trend is evident in global PCa rates, as well as in both low and highly developed regions [6].

There are considerable differences in PCa incidence and mortality across racial and ethnic groups. For example, PCa incidence and mortality rates are almost 3 times higher among black men than among white men in the United States [8]. Moreover, the number of PCa cases and deaths is much lower among Asian/Pacific Islanders, American Indian/Alaskan Natives and Hispanic men compared with non-Hispanic white men [8]. Further studies are needed to explain the reasons of these disparities, although some evidence suggests that they can be due to differences in access to diagnostic tests and treatments [20]. As mentioned above, the differences in the prevalence of multiple PCa genetic risk loci across racial/ethnic groups [21] may also be responsible for the disparities in incidence rates.

A family history of PCa is known to enhance the risk of PCa. Compared with men with no PCa family history, men with a father or brother diagnosed with PCa have a two to threefold higher risk of developing the disease, and the risk is nearly ninefold higher for

men with both [22]. A similar association has also been found for lethal PCa: men with a father or brother who died of PCa are at twofold higher risk of dying from this tumor compared with men without a PCa family history [23]. Interestingly, twin studies have highlighted that shared genetic factors are mainly responsible for PCa familial aggregation, with a heritability of almost 57% [24,25]. The more than 105 PCa risk loci identified across multiple studies explain about one third of the heritability [17,26]. Most of these germline risk loci do not correlate with PCa aggressiveness [27,28], indicating that inherited factors may be implicated in earlier prostate tumorigenesis.

1.3. Symptoms, diagnosis and prognosis of prostate cancer

The main signs and symptoms caused by PCa are:

- Weak or interrupted ("stop-and-go") flow of urine.
- Sudden urge to urinate.
- Frequent urination (especially at night).
- Trouble starting the flow of urine.
- Trouble emptying the bladder completely.
- Pain or burning while urinating.
- Blood in the urine or semen.
- A persistent pain in the back, hips or pelvis.
- Shortness of breath, fast heartbeat, dizziness or pale skin caused by anemia.

It should be noted that other conditions may cause the same symptoms, such as benign prostatic hyperplasia. Moreover, some cancers can also be asymptomatic, especially in their early stages of development [29].

PCa is generally diagnosed by transrectal ultrasound-guided biopsy, usually after abnormal PSA level changes and/or digital rectal examination. In particular, the PSA level at the time of diagnosis is also used as a prognostic factor, with higher PSA levels indicating a more aggressive disease. Similarly, the rate of PSA level increase is also an indicator of tumor aggressiveness: an increase of more than 2.0 ng/mL per year in PSA levels is often associated with a higher risk of PCa relapse after surgery or radiation therapy [29].

PCa stage is also determined using the tumor-nodes-metastasis (TNM) staging system [30]. In TNM staging, information about the tumor (T), nearby lymph nodes (N) and distant organ metastases (M) is combined, establishing the stage of the disease. In particular, the cancer stages are defined on the basis of tumor size and location:

- T stage corresponds to a tumor locally advanced.
- N stage represents the spread of PCa cells to the nearest lymph node through the lymphatic system.
- M stage correlates with the invasion by PCa cells of distant tissues and organs through the lymphatic or blood system. PCa frequently metastasizes to brain and bones.

Another staging system commonly used to determine PCa stage is the Gleason grade, based on the evaluation of the anatomy/histology of the PCa cells. If different cell types compose the tumor, its stage is established according to the Gleason score, based on the Gleason grade of the two most prevalent cell types. The Gleason score ranges from 2 to 10: tissues characterized by scores of 6 or less are supposed to look similar to normal tissues and to grow slowly; a score of 7 indicates an intermediate risk to develop an aggressive tumor; scores of 8 or higher describe poorly differentiated and metastatic cancers [30].

In summary, PCa is characterized by four progression stages, from I to IV, which are determined by TNM, Gleason score and serum PSA level (**fig. 2**). Early PCas (stages I and II) are localized and noninvasive; stage III tumors are larger, with a high risk to metastasize; advanced PCas (stage IV) have already colonized other body tissues [29,30].

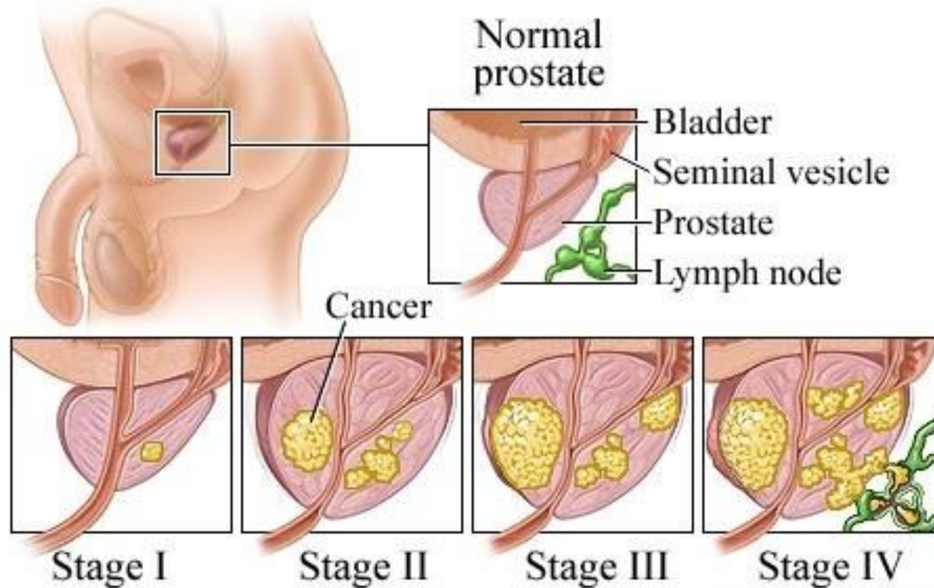

Fig. 2. *Prostate cancer staging.*

1.4. Molecular mechanisms in prostate cancer

Several oncosuppressor genes and oncogenes have been shown to be implicated in PCa development and progression.

1.4.1. Androgen receptor

A number of studies indicates that PCa growth and progression are driven by the androgen receptor (AR), a ligand-dependent transcription factor and member of the nuclear receptor family [31,32]. The AR is encoded by the AR gene located on the X chromosome at Xq11-12 and displays a N-terminal regulatory domain, a DNA-binding domain (DBD), a ligand-binding domain (LBD) and a C-terminal domain. In the absence of androgens, particularly dihydrotestosterone (DHT) and testosterone, it is complexed with chaperone proteins in the cell cytoplasm. After ligand binding, it translocates into the nucleus, where it homodimerizes due to the interactions of dedicated motifs present in the DBD and in the LBD. In the cell nucleus, the dimerized AR recognizes cognate DNA response elements in regulatory regions located in proximal or more distal intra- and intergenic regions of androgen target genes [33,34]. It then recruits a number of coregulator proteins and epigenetic factors to generate a transcriptionally active

complex able to upregulate downstream gene expression [31,32]. Downregulation of gene activity after interaction with corepressors has also been observed but is less well characterized [35] (**fig. 3**).

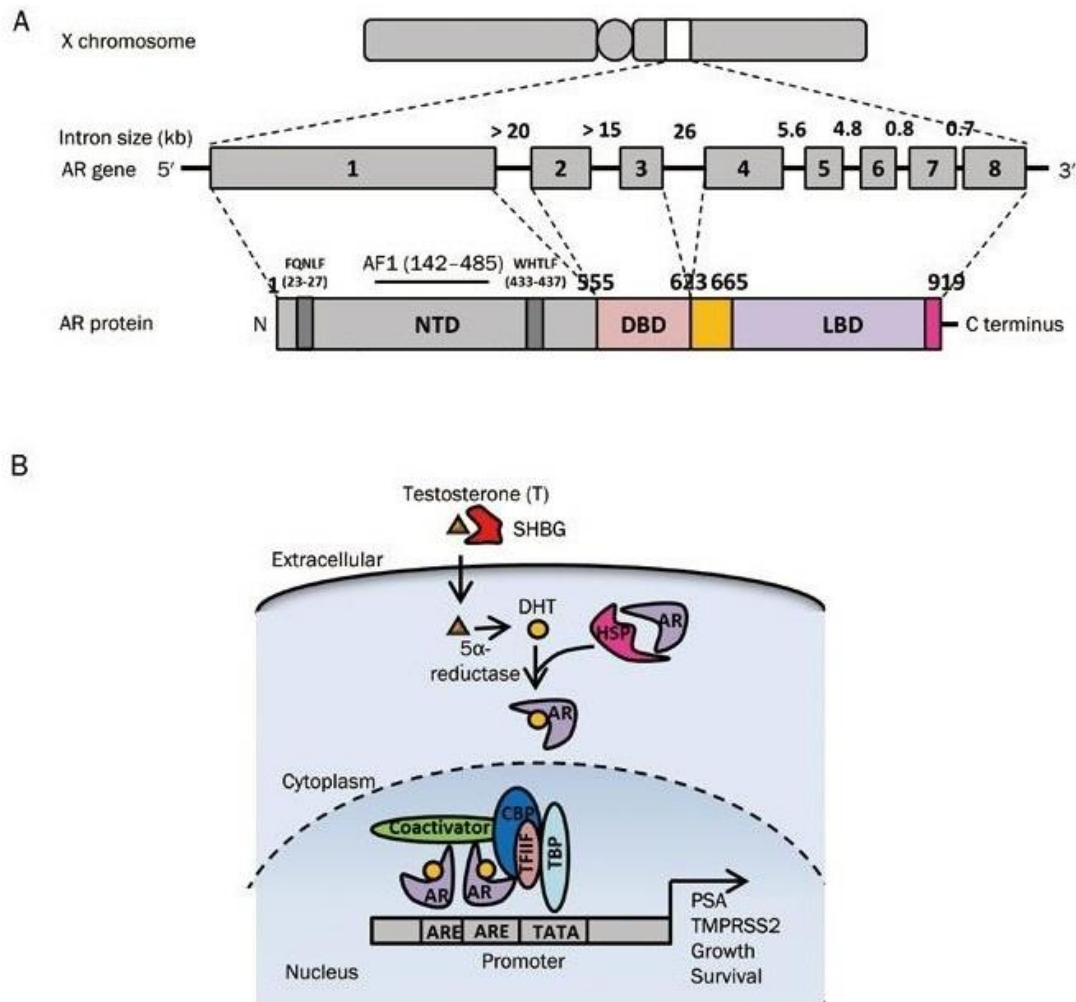

***Fig. 3**. **A**. Androgen receptor structure; **B**. Molecular mechanisms of androgen receptor function.*

As discussed in the following sections of this thesis, PCa often progresses towards a condition where its growth is castration resistant. One of the mechanisms underlying this change is an increase in the expression of AR in the tumor cell. In particular, it has been shown that 28% of cancers resistant to androgen deprivation therapy display AR upregulation due to amplification of its gene [36]. Another mechanism responsible for

PCa androgen-independent growth is ligand promiscuity, which results from AR gene mutations that cause amino acid substitutions in the LBD, leading to reduced specificity and selectivity for ligands: the most common of them are T877A, L701H, W741L and F876L. These mutant AR proteins bind to other steroid hormones, such as estrogen, progesterone and glucocorticoids, which can activate the AR signaling pathway and promote PCa progression [37]. In addition, certain antiandrogens can induce an agonist conformation in mutated ARs, leading to their activation rather than inhibition: this has been observed in patients undergoing flutamide treatment. AR activation via ligand-independent mechanisms represents the third mechanism of androgen-independent PCa development [38]. It has been found that tyrosine kinase receptor-activating ligands, such as insulin-like growth-factor-1 (IGF-1), keratinocyte growth factor (KGF) and epidermal growth factor (EGF), can activate the AR through the phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway. The AR can also be activated via crosstalk with other signaling pathways, such as the Src and Ack1 cascades [39-43]. Moreover, AR activation can also occur after binding to long non-coding RNAs, such as PCGEM1 and PRNCR1 [44]. Finally, various AR variants lacking the LBD have been recently reported: the AR NTD is constitutively active in the absence of the LBD and thus can promote castration resistant proliferation [45,46].

1.4.2. p53

The p53 gene is one of the most frequently mutated genes in human cancers. It is commonly known as “the guardian of genome”, due to its ability to block the synthetic phase of the cell cycle (phase S) and promote apoptosis in cells with damaged DNA. In primary PCa a relatively low incidence (10-20%) of p53 gene mutations has been observed, whereas it rises in advanced stages of the disease (42%) [47-49], being associated with high Gleason score [34], as well as with reduced survival after radical prostatectomy [50] and bone metastases [51].

1.4.3. PTEN

About 5-27% of localized and 30-60% of metastatic PCas display PTEN mutations [52-54]. This gene encodes a phospholipid phosphatase inhibiting the AKT signaling pathway, which plays a crucial role in cell cycle progression and cell proliferation [55]. PTEN can be found in normal prostatic epithelial cells and in cells with prostatic intraepithelial neoplasia (PIN), while its expression is downregulated in advanced cancers [34]. However, it has been demonstrated that common PTEN genetic variants do not significantly increase the risk of PCa [56]. On the other hand, PTEN modulates the expression of CDKN1B (p27), another oncosuppressor gene.

1.4.4. CDKN1B (p27)

p27 is a cyclin dependent kinase inhibitor, whose levels are known to be decreased in more aggressive PCas, particularly in those with a poor prognosis [57-60]. This gene is located on chromosome 12p12-3 and the somatic loss of its sequences has been reported in 23% of localized PCas, in 30% of regional lymph node metastases and in 47% of distant metastases [61]. Reduced p27 expression may be due to both CDKN1B alterations and PTEN downregulation.

1.4.5. NKX3.1

Homeobox protein NKX3.1 is a transcription factor that represses the PSA expression by binding to DNA [62]. This gene loss of function or deletion represents an early event in PCa [34], being present in androgen-sensitive cells but not in androgen-independent tumors [63]. In a recent study by Bowen *et al*. [64], NKX3.1 was shown to be absent in 20% of PIN lesions, 6% of localized PCas, 22% of advanced PCas, 34% of androgen-independent tumors and 78% of PCa metastases. Notably, the loss of this gene may be responsible for the increase in PSA levels observed during PCa progression.

1.4.6. Retinoblastoma protein

Retinoblastoma protein (Rb) suppresses the cell ability to replicate DNA, blocking its progression from the G1 (first gap phase) to S phase of the cell division cycle. Rb mutations are common in both clinically localized and advanced PCas [65,66], with at

least 50% of them observed in metastatic PCa [67,68]. This gene appears also to be involved in the regulation of prostate cell apoptosis, especially in response to androgens [69,70].

1.4.7. Glutathione S-transferase

Glutathione S-transferases (GST) are a family of enzymes that catalyze the conjugation of reduced glutathione to different substrates, preventing oxidant and electrophilic DNA damage [70]. In 96% of PCas, GSTP1 is hypermethylated and thus inactivated [71,72]. However, no risk for sporadic or familial PCa appears to be correlated with polymorphism in codon 105 of the pi variant (GSTP1 I105v) [73]. Interestingly, the calculation of GSTP1 hypermethylation can be effectively used to detect the presence of tumor even in small prostate tissue samples, which makes it a promising PCa diagnostic marker [74].

1.4.8. Kruppel-like factor 6

Kruppel-like factor 6 (KLF6) is a tumor suppressor gene that acts upregulating p21 in a p53-independent manner, thus suppressing cell growth [75]. Alterations of this gene, including deletions and loss of function, are present in a minority of high grade PCas [75,76].

1.4.9. Annexins

Annexins are calcium-binding proteins involved in several cellular processes, including cell motility and vesicle transport. They are significantly downregulated in PCa cell lines [77,78].

1.4.10. c-Myc

Myc is a family of regulator genes and proto-oncogenes encoding for transcription factors. The myc family consists of three related human genes: c-myc, l-myc (MYCL) and n-myc (MYCN). In cancer, c-myc is often constitutively expressed, promoting cell proliferation and transformation [34,79]. Its amplification and overexpression has been

reported in 8% of primary PCas and in about 30% of metastatic tumors [80,81]. Such overexpression is usually associated with high Gleason grade and poor prognosis in advanced PCa [81].

1.4.11. MX11

MX11 is a negative regulator of c-myc, which has been found to be mutated in PCa [82,83].

1.4.12. c-ErbB2 (Her-2 neu)

It is a member of the epidermal growth factor receptor (EGFR) family, encoding a plasma membrane-bound receptor tyrosine kinase. Its role in PCa progression is still a matter of debate: while some studies have highlighted that this gene is overexpressed in PCa, especially in the androgen-independent growing phase [84,85], other studies have not identified its amplification nor overexpression in PCa [86,87].

1.4.13. Bcl-2

This protein is generally expressed in PCa, whereas it is not present in the normal prostate [88]. It appears to be also involved in the progression towards metastatic PCa, because of its upregulation in the advanced stages of disease [89,90]. It has been proposed that androgen-mediated mechanisms may act through Bcl-2-related apoptotic pathways [91]. The overexpression of Bcl-2 prevents apoptosis in PCa cells [92,93].

1.4.14. Prostate stem cell antigen

Prostate stem cell antigen (PSCA) is a glycosylphosphatidylinositol (GPI)-anchored cell surface protein. In a recent study by Reiter *et al.*, it has been reported to be overexpressed in 80% of PCa specimens [94]. PSCA overexpression correlates with increasing Gleason score, disease stage and progression to androgen independence [95]. Notably, the administration of anti-PSCA monoclonal antibodies to mice successfully suppressed tumor growth and metastasis [96].

1.4.15. ERG and ETV1

These two genes are ETS transcription factors commonly upregulated in both primary and metastatic PCa tissues. They are activated by fusion to the TMPRSS2, a prostate-specific cell-surface serine protease, generating an androgen-responsive fusion oncoprotein [97,98].

1.4.16. Hepsin

This protein is a membrane bound serum protease playing a crucial role in cell proliferation. Its expression in PCa is associated with poor patient prognosis [99].

1.4.17. PIM1

It is a protein kinase known to be overexpressed in PCa [99].

1.4.18. ATFB1

ATFB1 is a transcription factor modulating α-fetoprotein expression [100], which appears to be involved in PCa growth. A study reported that 36% of the tumors tested had missense mutations inactivating the gene function [101].

1.4.19. A-Methyl Coenzyme A racemase

This enzyme is involved in fatty acid oxidation [102-104]. A recent study by Shand *et al.* has reported that approximately 88% of PCas were positive to A-Methyl Coenzyme A racemase (AMACR) staining [105]. It has also demonstrated 97% of sensitivity and 100% of specificity as PCa tissue marker in biopsy specimens [103].

1.4.20. CYP17

The cytochrome P-450c 17α enzyme, which is responsible for the synthesis of testosterone, is encoded by CYP17 allele. CYP17 allele mutations have been found in cases of sporadic and hereditary PCas [34].

1.4.21. SRD5A2

The allele SRD5A2 encodes for the enzyme 5α-reductase, which converts testosterone into 5-dihydrotestosterone (DHT) in the prostatic cell. Alleles that code for hyperactive enzymes have been shown to be associated with increased PCa risk [106].

1.4.22. CYP3A4

CYP3A4 is a member of the liver cytochrome P450 family of oxidizing enzymes, mainly involved in the metabolism of several xenobiotics, including various drugs. It plays an important role in the development of breast and prostate cancer by modulating the levels of sex hormone metabolites. In particular, CYP3A4 polymorphisms appear to confer a higher risk of PCa in men with benign prostatic hyperplasia. However, further studies are needed to confirm these data [107].

1.4.23. Vitamin D receptor

The vitamin D receptor (VDR) is an intracellular hormone receptor that specifically binds the active form of vitamin D (1, 25-dihydroxyvitamin D3 or calcitriol), which is known to induce differentiation and suppress the growth of PCa cells. A correlation between the best-characterized polymorphisms in the VDR variants (*i.e.*, Cdx2, Fokl, Bsml, Apal, Taql and the poly-A microsatellite) and PCa has been demonstrated [106].

1.4.24. c-Kit

c-Kit is a receptor tyrosine kinase implicated in intracellular signaling, as well as in occurrence of some cancers. Recently, it has been found to be expressed in PCa cells, contributing to disease progression [108].

1.4.25. STAT5

This protein regulates PCa cell growth and division, as well as cell death. Its phosphorylation correlates with high histological grade of PCa [109,110].

1.4.26. Insulin-like growth factor-1

Insulin-like growth factor-1 (IGF-1) is a hormone whose circulating levels have been strongly associated with PCa risk [111]. In particular, an IGF-I mRNA splice variant called IGF-IEc appears to regulate PCa growth via Ec peptide specific and IGF-IR/IR-independent signaling; interestingly, it was detected in PCa biopsies, where its expression correlates with tumor stage [112,113].

1.4.27. Interleukin-6

It is a cytokine implicated in the regulation of proliferation and differentiation of different cancer cells, including PCa cells [114]. In particular, it is known to modulate the STAT and/or mitogen activated protein kinase (MAPK) cascades. IL-6 levels are increased in sera of patients with advanced PCa. Moreover, PCa growth is accelerated after long-term exposure to IL-6 [115].

1.4.28. Transforming growth factor β

This multifunctional protein regulates proliferation and differentiation in several tumor types, including PCa [114]. Transforming growth factor β (TGF-β) promotes malignant transformation along with TGF-α, and it stimulates angiogenesis along with vascular endothelial growth factor (VEGF) [116]. Although TGF-β1 acts as a potent inhibitor of normal prostate epithelial cell growth and TGF-β1 receptor I and II dysfunction is associated with tumor aggressiveness, intracellular and serum TGF-β1 levels are elevated in PCa patients and further increased in patients with metastatic carcinoma [115].

1.5. Current therapies

In about 90% of cases, PCa is still organ-confined or only locally advanced at diagnosis, which makes it effectively treatable with prostatectomy or local radiotherapy. However, most of patients usually experience disease progression; in this phase, in which tumor growth is dependent on the presence of androgens, androgen-deprivation therapy, aimed at blocking androgen secretion/activity, represents the most effective treatment. This therapy includes chemical castration, which can be achieved by GnRH agonists,

given either alone or in combination with antiandrogens. However, after an excellent initial response, relapse occurs in most patients within a median of 2–3 years, and the tumor progresses towards a condition of resistance to castration [castration-resistant prostate cancer (CRPC)]. For CRPC patients the therapeutic options are still very limited, since taxane-based (*i.e.*, docetaxel) treatment and immunotherapy, as well as the novel therapies with enzalutamide and abiraterone, usually provide a progression-free survival of a few months. The current treatment options for PCa patients are summarized in **fig. 4**.

1.5.1. Surgery

Surgery, particularly radical prostatectomy and pelvic lymphadenectomy, is usually suggested in case of high-risk locally advanced PCa [117].

Traditionally, the use of radical prostatectomy for high-risk PCa treatment has been discouraged due to its several side effects, including high rates of positive surgical margins, risk of lymph node metastasis and high rates of PSA recurrence. However, surgery has been demonstrated to be more beneficial than watchful waiting in terms of mortality and risk of both local progression and metastasis [118]. Patients with a biopsy Gleason score ≤ 8 and the serum PSA level < 20 ng/ml are usually recommended to undergo surgery [119]; these criteria are currently suggested by the European Urology Association [120] for the treatment of advanced PCa [121].

Pelvic lymphadenectomy is generally performed during radical prostatectomy in case of high-risk PCa [118], since 15-40% of nodes would generally have positive results [122]. In this regard, pelvic lymphadenectomy is the most reliable strategy to detect the lymph node metastases in PCa; however, its therapeutic potential is still a matter of debate [123].

1.5.2. Radiation therapy

After surgery, radiotherapy is considered as the second major therapeutic option for the treatment of localized high-risk PCa. In the last decades, external-beam radiotherapy

and brachytherapy have undergone considerable clinical and technological development and are now widely used in PCa management [124].

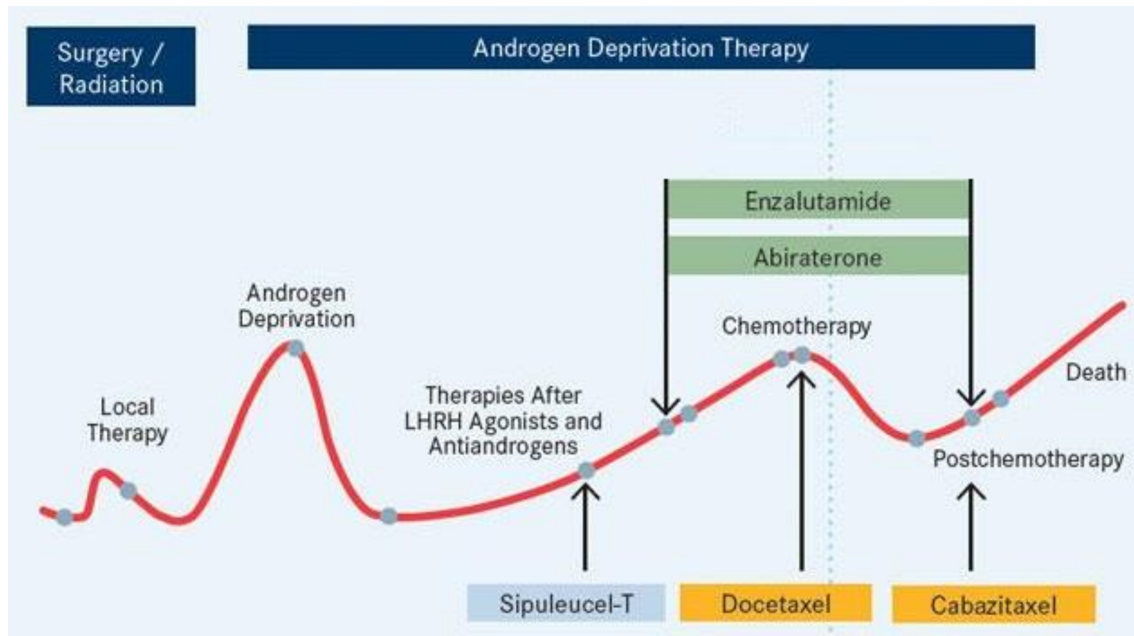

Fig. 4. *Treatment landscape in prostate cancer.*

Patients without distant metastases and a life expectancy of at least 5-10 years can undergo external-beam radiotherapy [125]. A radiation dose of at least 74 Gy should be the standard of care for all men with localized PCa who choose treatment with external-beam radiotherapy [124]. However, the optimal dose of external-beam radiotherapy has not yet been established. Some randomized trials have highlighted the benefits of a dose escalation up to the total dose of 76-78 Gy, while others have shown the advantage of the addition of adjuvant antiandrogen therapy to external-beam radiotherapy for patients with locally advanced cancers. For increasing survival and reducing metastases risk, adjuvant postprostatectomy external-beam radiotherapy of the prostatic fossa with doses in the range of 60-66 Gy has also been suggested [125].

High-dose rate brachytherapy involves a temporary insertion of applicators into the prostate, in order to target different zones of the gland and minimize the dose of radiation to the bladder and the bowel. It can be used as monotherapy or in combination

with external-beam radiotherapy, especially for patients with more locally advanced disease [126].

In low-dose rate brachytherapy, radioactive seeds with a half-life of two months are permanently put into the prostate under a general anaesthetic or spinal anaesthetic. In a small randomized trial, surgery and low-dose rate brachytherapy produced equivalent outcomes, with a 5-year biochemical progression-free survival of 91.0% by surgery versus 91.7% by brachytherapy; on the contrary, they differed for the severity of the side effects, particularly as regards urinary and erective disorders [127]. Interestingly, these two treatments have similar cost profile in France [128].

1.5.3. Proton beam therapy

Proton beam therapy involves the use of a particle accelerator to target the tumor with a beam of protons, localizing the radiation dosage more precisely when compared to other types of radiotherapy. In particular, proton beam therapy offers an excellent dose distribution, with no exit dose. For these reasons, it represents an excellent choice for the treatment of PCa [129]. In a recent phase III trial [130], an increased dose with an external proton beam of 12.5% to 75.6% CGE (Cobalt Gray Equivalent) significantly improved local control of poorly differentiated PCas, when compared to a conventional dose. Over the last ten years, proton beam therapy has effectively increased the survival rate among PCa patients [131].

1.5.4. Cryosurgery

Cryosurgery consists of the application of extreme cold to an abnormal tissue, in order to destroy it. In this context, the supercooled liquid is sprayed on the tumor by using liquid nitrogen as the cooling solution. Focal cryotherapy has recently emerged as an effective therapeutic option for the treatment of localized low-risk PCa [132]. In particular, the efficacy and safety of PCa targeted cryoablation was reported in a series of 590 consecutive patients [133]. Compared to brachytherapy, cryotherapy was shown to be less irritative, with lower complication rates and improved urinary function after treatment [134]. In a randomized trial aimed at comparing cryoablation with external-

beam radiotherapy in PCa patients, cryosurgery was found to produce better outcomes [135].

1.5.5. Hormonal therapy

Androgen deprivation therapy is regarded as the initial treatment for advanced PCa. The dramatic clinical effects obtained by suppressing serum testosterone levels in men with metastatic PCa were reported for the first time by Huggins *et al*. in 1941 [136]. Indeed, inhibition of various hormones, receptors or enzymes involved in the androgen synthesis pathway is at the basis of this treatment.

GnRH agonists

GnRH agonists, including goserelin, leuprorelin and triptorelin, induce castrate levels of testosterone by binding to their associated receptors in the anterior pituitary. This leads to the downregulation of the receptors, decreasing the release of luteinising hormone (LH) from the pituitary and subsequently reducing the production of testosterone by Leydig cells in testes. Notably, castrate levels of testosterone (<1.74 nmol/L (< 50 ng/dL)) are reached within four weeks. GnRH agonist side effects include hot flashes, loss of libido, erectile dysfunction, depression, muscle wasting, anemia and osteoporosis, as well as the flare phenomenon, where testosterone levels are initially increased due to GnRH receptor stimulation [137].

Anti-androgens

As mentioned above, the initial stimulation of the GnRH receptors may lead to an initial flare-up of testosterone level, lasting up to 10 days. For this reason, anti-androgens are often administered among with GnRH agonists. This is a class of drugs that act mainly by inhibiting the androgen receptor signaling, sometimes by competing with testosterone and DHT for receptor binding. Inhibition of the AR cascade leads to apoptosis and suppression of PCa growth. There are two sub-classes of anti-androgens: steroidal (cyproterone acetate) and non-steroidal (bicalutamide, nilutamide, and flutamide) [137].

Newer drugs

Abiraterone acetate is an irreversible inhibitor of CYP17A1, a 17–20 lyase and 17-α hydroxylase of the cytochrome P450 family, which converts pregnanes into steroid hormones, including androgen precursors [138]. Therefore, it can successfully suppress androgen synthesis in the testis and adrenal glands, as well as in PCa, thus blocking its growth. The main adverse events associated with abiraterone acetate treatment are related to the increase in the mineralocorticoid levels due to CYP17A1 inhibition; for this reason, prednisone or prednisolone are generally concomitantly administered. Abiraterone acetate was first approved by the Food and Drug Administration (FDA) in 2011 for late-stage CRPC patients, and then in 2012 also for use prior to chemotherapy [138]. It is currently being tested in combination with various therapies, and it has recently demonstrated improved effectiveness when given along with androgen deprivation therapy in patients with locally advanced PCa [139-141]. Other CYP17A1 inhibitors, including orteronel and galeterone, have been evaluated in clinical studies but did not reach their primary endpoint [142].

Enzalutamide is a second-generation, competitive oral AR antagonist approved by the FDA for metastatic CRPC treatment post- and pre-chemotherapy in 2012 and 2014, respectively [143]. In addition, it significantly enhances metastasis-free survival in high-risk non-metastatic CRPC patients. Toxic effects due to the penetration of the drug in the brain have been observed [144,145]. Combinations of enzalutamide with other drugs already approved for early- or late-stage PCa, including abiraterone acetate, docetaxel or radium-223 dichloride, are now under intensive study [146], and a phase III combination study with the PD-L1 antibody atezolizumab has also started [147].

Apalutamide is an oral AR antagonist structurally related to enzalutamide [148], which has recently been approved for non-metastatic CRPC treatment, based on a 24-month longer metastasis-free survival [149]. Common side effects include rash, fatigue, arthralgia, weight loss, fails and fracture. Various clinical trials are currently evaluating the effectiveness and safety of combinations of apalutamide with GnRH ligands and abiraterone acetate.

Darolutamide is a second-generation competitive oral AR antagonist with a novel chemical structure [150,151]. Unlike other AR antagonists, darolutamide does not penetrate the blood-brain barrier, displaying low toxicity [152]. Phase III studies are currently ongoing for darolutamide in addition to standard androgen deprivation therapy plus docetaxel in CRPC.

A strategy to overcome resistance associated with AR ligand-binding domain mutations is to target other AR regions. In this regard, a non-competitive AR antagonist binding to the N-terminal domain, named ralaniten acetate (EPI-506), has been tested in clinical phase I/II for the treatment of metastatic CRPC [153]. Other molecules targeting the AR binding function site 3 or DNA-binding domain have been identified [154,155].

1.5.6. Chemotherapy

The use of chemotherapy in CRPC patients has demonstrated significant improvements in pain and quality of life, as well as reductions in PSA levels [156]. The most used chemotherapeutic drugs include mitoxantrone, doxorubicin, vinblastine, paclitaxel and docetaxel.

Mitoxantrone is an anthracenedione anti-tumor agent. When given in combination with prednisone, it improves the quality of life but not the survival rate in patients with CRPC, thus being used as a second-line treatment [157].

Taxol is a well-known cytoskeletal drug that targets tubulin. In particular, it stabilizes the cell microtubules and prevents their disassembly, thus blocking the progression of mitosis and prolonging the activation of the mitotic checkpoint, ultimately leading to apoptosis or reversion to the G0-phase of the cell cycle without cell division. Several studies pointed out that the survival rate of men with CRPC is significantly higher after the treatment with docetaxel and prednisone than that with mitoxantrone and prednisone [158].

Anthracyclines, doxorubicin or epirubicin, either alone or in combination with other agents, have been used extensively in the CRPC management, but the outcomes are still controversial [159].

1.5.7. Immunotherapy

Sipuleucel-T is a cell-based cancer immunotherapy, which was approved for the treatment of CRPC in 2010 [160]. It involves the extraction of peripheral blood mononuclear cells from the patient by leukapheresis, followed by their incubation with recombinant prostatic acid phosphatase (PAP) coupled to granulocyte-macrophage colony-stimulating factor for maturation of antigen-presenting cells. The activated product is then reinfused into the patient in three courses at two-week intervals in order to eliminate PCa cells expressing high levels of PAP. Long-term clinical benefits with only mild, manageable side-effects have been reported [160]; however, till now the use of sipuleucel-T has been limited due to its high costs and complex procedure [161,162].

PCa has a relatively low proportion of cancer-specific neoantigens, indicating that it may be less susceptible to treatments with immune checkpoint inhibitors [163,164]. However, based on the several benefits demonstrated by therapies with immune checkpoint inhibitors in many tumor types, the potential of immunotherapy has also been investigated in PCa [165,166]. The anti-CTLA4 antibody ipilimumab has shown some promising results in PCa patients, causing a complete tumor remission in some cases, although no enhanced overall survival was confirmed in larger studies [167,168]. Durable responses to the anti-PD-1 antibody pembrolizumab have been reported in patients with CRPC [165,166]. Importantly, pembrolizumab has been recently approved for the treatment of mismatch repair-deficient solid tumors, so that CRPC patients belonging to this group can be treated with this drug [169]. Coming to anti-PD-L1 antibodies, early clinical studies with atezolizumab, durvalumab and avelumab are ongoing for CRPC treatment [165].

1.5.8. Targeted alpha therapy approach

A targeted alpha therapy approach based on the intravenous administration of radium-223 dichloride has been approved for CRPC patients with bone metastases by the FDA in 2013. Radium-223 dichloride is able to mimic calcium, thus penetrating in osteoblastic bone metastases and binding to hydroxyapatite, a major component of bone [170,171].

In vivo experiments performed in PCa-bearing mice have pointed out that, after being taken up into the bones, radium-223 dichloride induces cytotoxicity in tumor cells, osteoclasts and disease-promoting osteoblasts by triggering extensive DNA damage and fragmentation [172].

When given to CRPC patients, radium-223 dichloride significantly improves overall survival irrespective of prior docetaxel use, alleviating bone metastases-related pain without causing any toxicity [173-175]. Combination studies with enzalutamide, olaparib and niraparib are ongoing [174]. Moreover, radium-223 dichloride has been found to trigger T cell-mediated lysis in different tumor types, including PCa [176]. Thus, combination treatments with different immunotherapies, including the checkpoint inhibitors pembrolizumab and atezolizumab, are currently being tested.

1.5.9. Dietary strategies

Like numerous other diseases, cancer development can be influenced by the interactions between individual genetic susceptibility and life style background. Thus, changes in diet may represent an effective strategy to prevent cancer, because some dietary factors may contribute to tumor progression, while others could reduce cancer growth.

Dietary fat and cholesterol play a crucial role in PCa development, thus avoiding them may help to control or prevent this disease [177]. In particular, ω-6 polyunsaturated fatty acids have been reported to exert pro-tumor effects during prostate carcinogenesis, while ω-3 polyunsaturated fatty acid-rich oils are known to suppress PCa growth [178].

A no-carbohydrate ketogenic diet has been shown to significantly reduce PCa progression and to prolong survival in xenograft model mice injected with PCa cells [179].

A vitamin D intake seems to reduce the risk of PCa, also improving the survival of patients with this disease. Similarly, vitamin B-6 has been found to enhance PCa survival among men with a diagnosis of localized-stage disease [180,181].

As discussed in the following chapter of this thesis, several nutraceutical compounds are now under intense study for the treatment of different types of tumor, including PCa.

2. TOCOTRIENOLS

2.1. Nutraceuticals

The term “nutraceutical” was coined in 1989 by Dr. Stephen De Felice, MD founder and chairman of the Foundation for Innovation in Medicine, who defined it as a “food, or parts of a food, that provide medical or health benefits, including the prevention and treatment of disease”. Thus, nutraceuticals may refer to naturally nutrient-rich or biologically active foods and herbs, such as blueberries or soybeans, or they may be specific food components or isolated nutrients and phytochemicals with medicinal properties, such as polyphenols, flavonoids and carotenoids [182].

Numerous phytochemicals have been found to exert potent anti-cancer activities, and some of them, such as taxanes, vinca alkaloids and podophyllotoxin analogues, are currently used in chemotherapy.

Epigallocatechin gallate (EGCG), the ester of epigallocatechin and gallic acid, belongs to the catechin subclass of flavonoids. It is the major component of green tea and is a potent free-radical scavenger and antioxidant. In addition, several ongoing clinical trials have demonstrated its ability to synergistically increase the efficacy of conventional chemotherapy against PCa [183].

Curcumin is a polyphenol obtained from *Curcuma longa*, commonly known as turmeric. It is nontoxic and characterized by many therapeutic properties, particularly by antioxidant, anti-inflammatory and anti-microbial activities. Moreover, it exerts significant anti-tumor effects in both PCa cells and mouse models [183].

Resveratrol (trans-3,4’,5-trihydroxystilbene) is a grape-derived polyphenol that has been intensively studied for its chemopreventive potential. In the case of PCa, resveratrol has been found to suppress the proliferation of cancer cells [184].

Lycopene is a carotenoid naturally occurring in many red fruits and vegetables, such as tomatoes, watermelon and pink grapefruit. A combination of lycopene, vitamin E and selenium can inhibit PCa development and increase disease-free survival [183].

Genistein (4',5,7-trihydroxyisoflavone) is a phytoestrogen commonly found in soybeans. Its administration has been correlated with a decrease in the incidence of PCas [185].

2.2. Triggering cell death with natural compounds

Accumulating evidence has highlighted the ability of numerous natural compounds to specifically trigger different pro-death systems, such as apoptosis, paraptosis, endoplasmic reticulum (ER) stress and autophagy, in a variety of cancer cells, including PCa models.

2.2.1. Apoptosis

The term "apoptosis", from the Greek "*απο*" and "*πτωσιζ*" ("dropping off"), refers to a highly selective process in which a cell commits suicide after receiving certain stimuli. It was first described by Kerr *et al.* in 1972 and it was shown to play a key role in both physiological and pathological conditions [186].

Morphology of Apoptosis

The main morphological changes associated with apoptotic cell death have been highlighted by light and electron microscopy [187]. During the early stages of apoptosis, cells undergo shrinkage and pyknosis, appearing smaller in size and with a dense cytoplasm, tightly packed organelles and condensed chromatin within the nucleus. In particular, cells stained with hematoxylin and eosin are round- or oval-shaped and show a dark eosinophilic cytoplasm and dense purple chromatin fragments aggregating peripherally under the nuclear membrane. Subsequently, extensive plasma membrane blebbing is observed, accompanied by karyorrhexis, the rupture of nuclear membrane and the release and degradation of its content, and budding, a process where the cell is fragmented into membrane-bound vesicles called "apoptotic bodies". These bodies are then engulfed by macrophages and other phagocytic cells before they can release their content into the surrounding interstitial tissue. In particular, the removal of dying cells

by phagocytes occurs in an orderly manner without eliciting an inflammatory response. It should also be noted that apoptosis involves only single cells or small clusters of cells.

Apoptotic pathways

The two best-characterized apoptotic pathways are the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway [188,189] (**fig. 5**).

The extrinsic pathway is mediated by cell surface death receptors belonging to the tumor necrosis factor receptor superfamily (TNFRS). They possess an extracellular cysteine-rich domain, a transmembrane domain and a cytoplasmic domain called “death domain”, which plays a crucial role in transmitting the death stimulus from the cell surface to the intracellular molecular pathways. To date, the best-known ligand/death receptor systems are FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5.

The extrinsic pathway is activated by the binding of a specific death receptor with its extracellular ligand. The obtained composite then recruits death domain-containing protein (FADD) and pro-caspase-8, forming the death-inducing signaling complex (DISC). This results in the activation of pro-caspase-8, which leads to the cleavage of pro-caspase-3, the main enzyme responsible for the execution of the apoptotic process [190,191].

The intrinsic pathway is regulated by mitochondrial enzymes, particularly by those belonging to the Bcl-2 family [192]. This group is divided into two different sub-categories of proteins, the pro-apoptotic proteins (*e.g.*, Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) and the anti-apoptotic proteins (*e.g.*, Bcl-2, Bcl-X_L, Bcl-W, Bfl-1 and Mcl-1). While the anti-apoptotic proteins block cell death by preventing the mitochondrial release of cytochrome *c*, the pro-apoptotic proteins stimulate such release, so the balance between them determines whether apoptosis would be initiated or not. In particular, when the pro-/anti-apoptotic protein ratio is increased, outer mitochondrial membranes become permeable to internal cytochrome *c*, which is released into the cytosol. Cytochrome *c* then recruits apoptotic protease activating factor-1 (Apaf-1) and pro-caspase-9 to compose the so-called “apoptosome”, which triggers a caspase-9/3 signaling cascade, culminating in apoptosis [193–195].

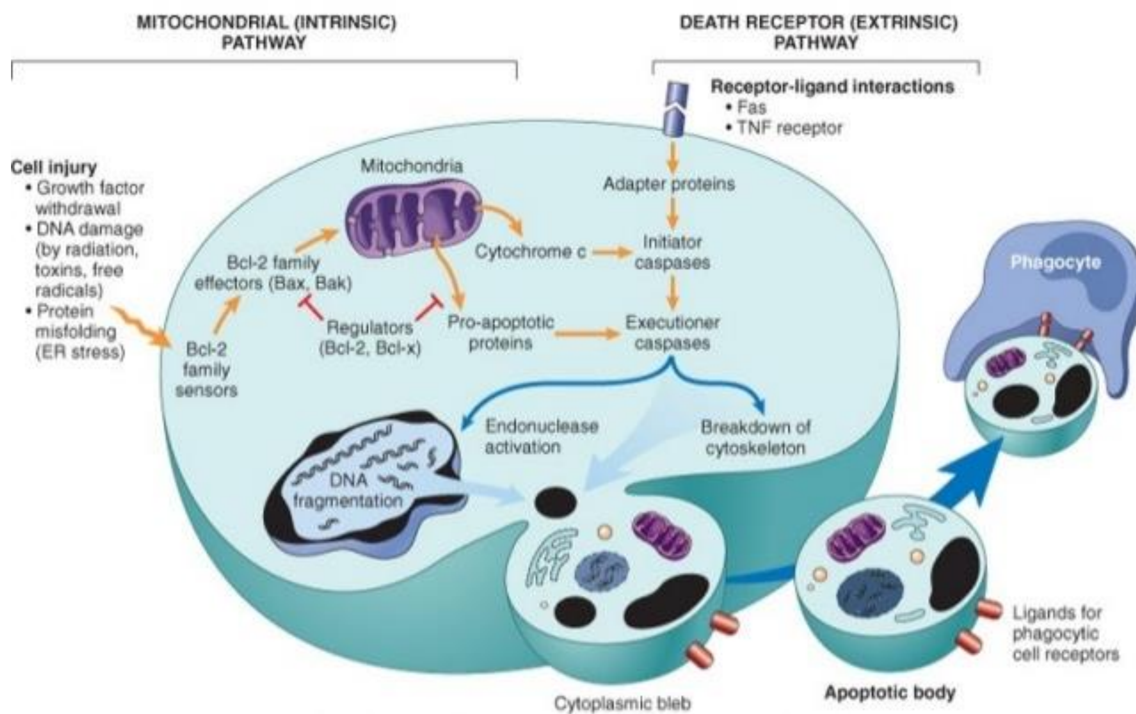

***Fig. 5**. Main features of apoptosis.*

The caspase cascade

Caspases (cysteine-aspartic proteases, cysteine aspartases or cysteine-dependent aspartate-directed proteases) are a family of protease enzymes implicated in apoptosis and inflammation. They are synthesized as inactive zymogens called "pro-caspases", which are activated by post-translational modification only after proper stimulation, allowing a rapid and tight regulation of the enzyme. In particular, the caspase activation mechanism involves dimerization and often oligomerization of the pro-enzyme, followed by its cleavage into a small subunit and large subunit. The two subunits then associate with each other to form an active heterodimer complex.

Caspases have been broadly classified by their roles in apoptosis (caspase-2, -3, -6, -7, -8, -9 and -10 in mammals) and in inflammation (caspase-1, -4, -5, -12 in humans and caspase-1, -11, and -12 in mice). Apoptotic caspases have been subdivided on the basis of their mechanism of action into initiator caspases (caspase-8 and -9) or executioner caspases (caspase-3, -6, and -7). The other caspases that have been identified include caspase-13, which is suggested to be a bovine gene, and caspase-14, which is selectively

expressed in the epidermis and the hair follicles, where it contributes to epidermal differentiation.

Caspases have proteolytic activity and are able to cleave proteins at aspartic acid residues. Common caspase targets are: mediators and regulators of apoptosis (*e.g.*, Bid, Bcl-2 and Bcl-Xl); structural proteins (*e.g.*, nuclear lamins, fodrin, gelsolin, keratins 18 and 19, vimentin, β-catenin and plakoglobin γ-catenin); cellular DNA repair proteins (DNA- dependent protein kinase (DNA-PK), Rad51, ATM serine/threonine kinase, poly(ADP-ribose) polymerase (PARP)); cell cycle-related proteins (Cdc27, Wee1, Rb and the two Cdk inhibitors p21CIP1 and p27KIP1) [196,197].

Other biochemical features of apoptosis

Another biochemical hallmark of apoptosis is the expression of cell surface markers ("eat me" signals) that allows the recognition of dying cells by phagocytes, leading to a quick phagocytosis with minimal compromise to the surrounding tissue. This is achieved by the externalization of phosphatidylserine. In fact, while this phospholipid is normally confined to the cytoplasmic leaflet of the plasma membrane by a flippase, it is rapidly exposed on the cell surface by a scramblase in case of apoptosis. In addition to phosphatidylserine, recent studies have shown that other proteins are also externalized on the cell surface during the apoptotic cascade, such as Annexin I, a protein usually implicated in the regulation of the anti-inflammatory effects of glucocorticoids, and calreticulin, a Ca^{2+}-binding chaperone that promotes protein folding and quality control in the ER lumen [198].

Apoptosis in cancer

Evasion of cell death is one of the main changes that occur in a cell during its malignant transformation. Interestingly, there are several mechanisms by which a tumor cell can acquire apoptosis resistance. For instance, downregulation and loss of function of different death receptors have been found in various tumor types, leading to impairment of the apoptotic extrinsic signaling. Moreover, a disrupted balance of pro-apoptotic and anti-apoptotic proteins has been observed in several cancers: this appears to involve not only the Bcl-2 protein family but also p53 and the inhibitor of apoptosis

proteins (IAPs) [199]. Hence, triggering alternative pro-death pathways could represent an effective strategy to eliminate cancer cells unaffected by the apoptotic cascade [200]. In this context, paraptosis, ER stress and autophagy have recently gained increasing interest, mainly due to their involvement in the anti-cancer activity of various natural compounds.

2.2.2. Paraptosis

Paraptosis was first reported by Sperandio *et al.* in 2000 [201]. It is a type of programmed cell death displaying cytoplasmic vacuolization, usually consisting in mitochondrial and/or ER swelling. It requires protein synthesis and can be successfully blocked by the translation inhibitor cycloheximide. Unlike apoptosis, paraptosis does not involve the activation of caspases or the formation of apoptotic bodies; indeed, it is not affected by caspase inhibitors or overexpression of Bcl-2-like anti-apoptotic proteins. On the other hand, paraptosis has been demonstrated to be dependent on mitogen-activated protein kinase (MAPK) family members, such as mitogen-activated protein kinase kinase 2 (MEK-2), c-Jun N-terminal protein kinase 1 (JNK1) and p38, and it can be inhibited by the multifunctional adapter protein AIP-1/Alix [202]. As shown in the following paragraphs of this review, it is often accompanied by an alteration of Ca^{2+} and redox homeostasis, as well as by ER stress, a condition where unfolded and misfolded proteins accumulate in the ER lumen, ultimately leading to the activation of pro-death processes (**fig. 6**). However, these features are not always present in cells undergoing paraptosis. Hence, the term “paraptosis-like cell death” has been coined, to describe those types of programmed cell death resembling paraptosis but lacking one or more of its common characteristics [203].

Paraptosis is known to occur during neural development, and it has also been observed in different neurodegenerative diseases and neurological disorders [204]. Moreover, it appears to be involved in retinal pathologies: it is activated after both glucocorticoid treatment [205,206] and reperfusion injury [207], as well as in the early phases of glaucoma [208].

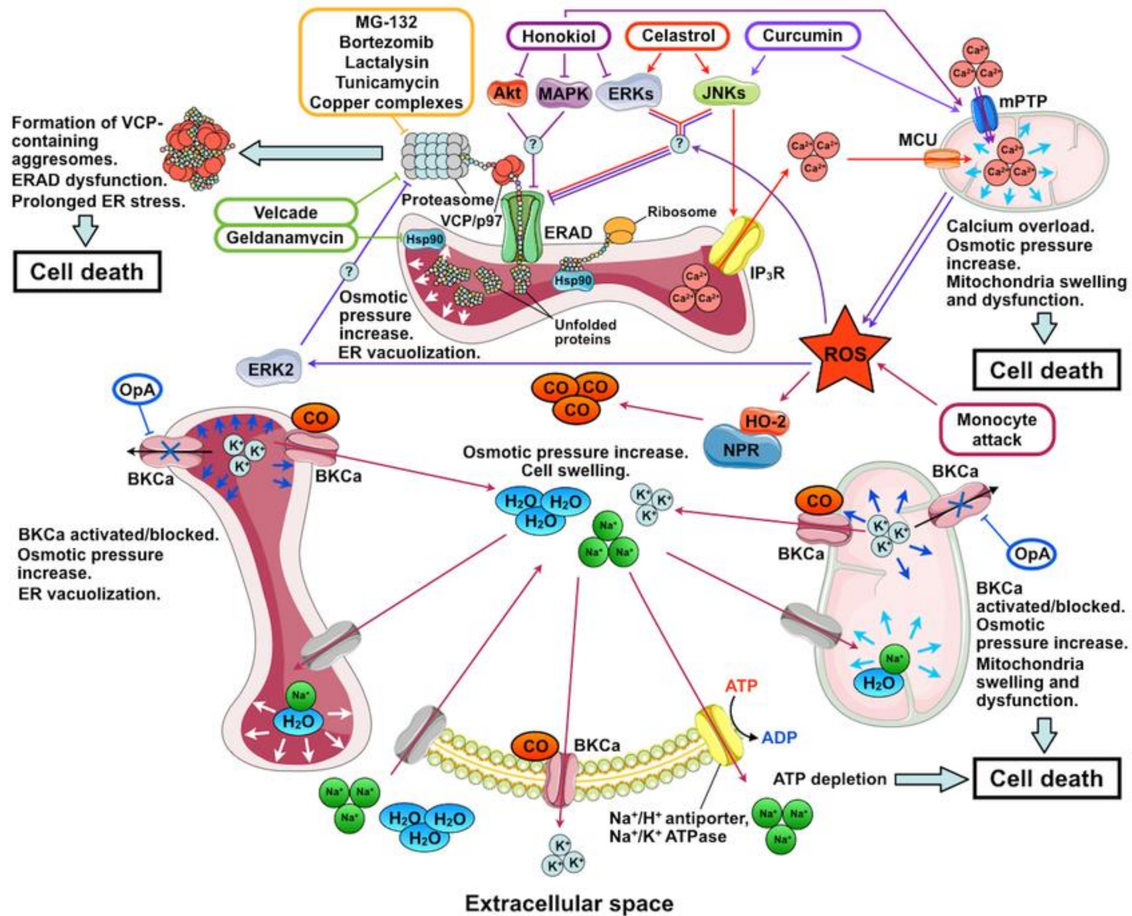

Fig. 6. Main features and inducers of paraptosis.

Established mediators of paraptotic cell death are insulin-like growth factor 1 receptor (IGFR-1) [202], the neuropeptide substance P [209], TAJ/TROY, an orphan TNF receptor family member [210], epidermal growth factor (EGF) [211,212] and adenine nucleotide translocase 1 (ANT1), a multitask protein implicated in cell proliferation and metabolism [213]. Moreover, both membrane cholesterol and heme homeostasis have been demonstrated to play a crucial role in the regulation of paraptosis [214,215]. Finally, human glioma cells retrovirally transduced with the human gene for the membrane form of macrophage colony-stimulating factor (mM-CSF) were found to be eliminated by human monocytes through the paraptotic pathway [216–218].

Coming to cancer treatment, many natural compounds have been shown to cause paraptosis in various human cancer cell lines. Among them, taxol, cyclosporine A, tunicamycin, procyanidins, curcumin, honokiol, ginsenosides, tocotrienols, celastrol,

ophobiolin A, hesperidin, morusin, 6-shogaol, chalcomoracin, gambogic acid, plumbagin, 8-p-hdroxybenzoyl tovarol, *cis*-nerolidol, manumycin A, DL-selenocystine, 15-deoxy-Δ12,14-prostaglandin J2, yessotoxin and 1-desulfoyessotoxin have shown promise as pro-paraptotic agents [219].

2.2.3. Endoplasmic reticulum stress

The ER is a network of membranous tubules and sacs within the cytoplasm of all eukaryotic cells, continuous with the nuclear membrane. It is mainly involved in protein synthesis, folding, post-translational modifications and delivery, and it is also one of the most important Ca^{2+} stores. In particular, the maintenance of the homeostasis of this ion is warranted by the sarco/ER Ca^{2+}-ATPase (SERCA), and it is fundamental since many ER chaperone proteins implicated in protein folding, such as BiP, calnexin, calreticulin and PDI, are Ca^{2+} dependent [220,221].

ER functions can be altered by many signals: physiologic or pathologic stimuli might increase the demand for protein synthesis, while stressful events might disrupt protein folding. For instance, an increment in protein synthesis is often observed in overproliferating tumor cells: if it is not coupled with an efficient protein folding, an excess of unfolded proteins accumulates in the ER. On the other hand, various stressful conditions, including hypoxia, glucose deprivation and oxidative stress, may cause an unfavorable environment for the maintenance of protein homeostasis. Cells initially respond with a defensive unfolded protein response (UPR), accompanied by an increase in the ER protein folding capacity and in the ER-associated protein degradation (ERAD) machinery; however, in case of severe or prolonged stress, unfolded and misfolded proteins may exceed the ER capacity and accumulate in its lumen, leading to the activation of a set of pro-death programs [220,221].

Three major proteins are known to act as stress sensors in the ER (**fig. 7**): double-stranded RNA-dependent protein kinase PKR-like ER kinase (PERK), inositol-requiring 1α (IRE1α) and activating transcription factor 6 (ATF6). Each of these proteins possesses an ER luminal domain able to detect the presence of unfolded proteins in the ER, an ER-transmembrane domain and a cytosolic domain that transduces the signals to the

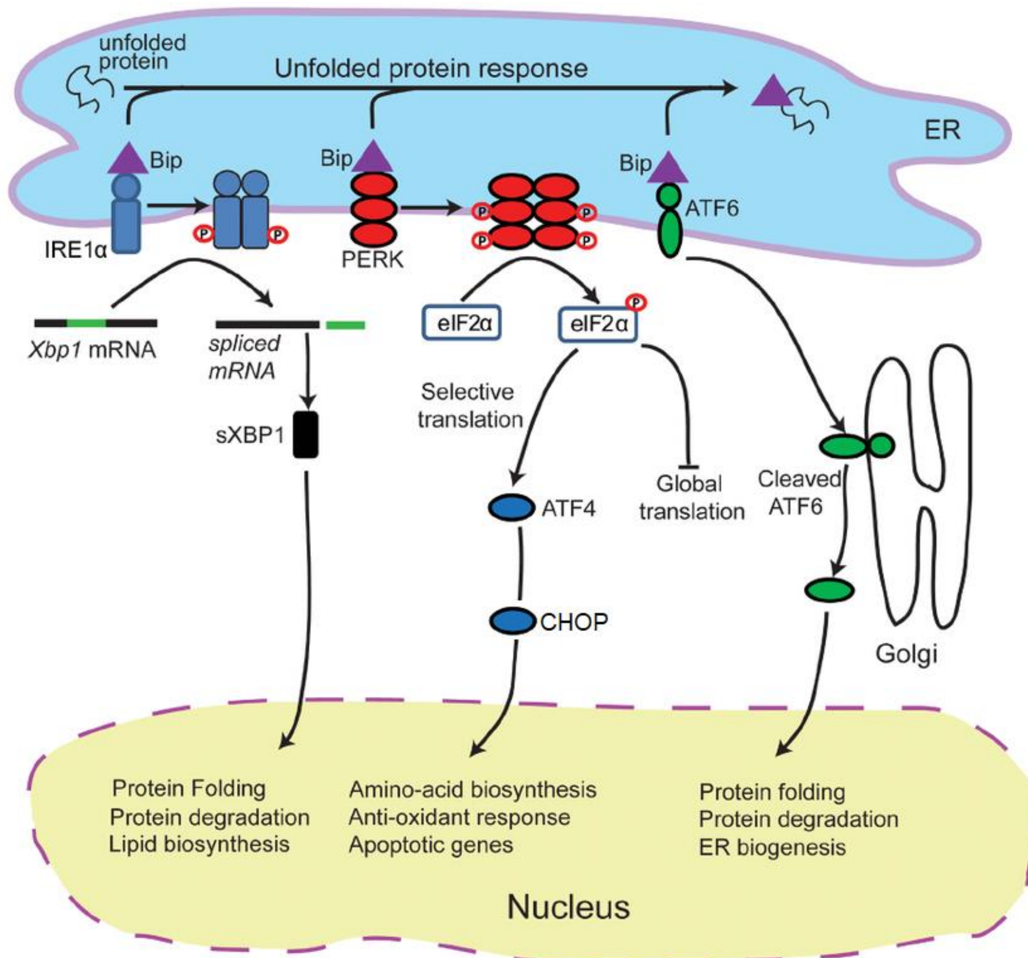

***Fig. 7**. ER stress.*

cytoplasm. In normal physiological conditions, these sensors are inactivated by the association with the chaperon protein immunoglobulin-heavy-chain-binding protein (BiP, also known as GRP78). However, in case of unfolded protein overload, BiP dissociates from these complexes to bind to nascent polypeptides and promote their folding, thus triggering the activation of the three sensors and the induction of UPR: several emergency systems are activated in order to re-equilibrate protein homeostasis [220]. In particular, the dissociation of BiP from PERK induces the homodimerization and subsequent autophosphorylation of the kinase, that in turn phosphorylates the eukaryotic translation-initiation factor 2α (eIF2α), which blocks protein synthesis. Similarly, the dissociation of BiP from IRE1α leads to its activation via dimerization and autophosphorylation. IRE1α displays an endoribonuclease (RNase) domain, which

catalyzes the removal of a 26-nucleotide intron from the X box binding protein 1 (XBP1) mRNA: the spliced XBP1 (sXBP1) triggers the transcription of numerous UPR target genes. The RNase domain is also responsible for the cleavage of other mRNAs, further contributing to the downregulation of protein translation during ER stress. After BiP release, ATF6 is translocated to the Golgi and cleaved by the proteases S1P and S2P, generating a functional fragment that induces the transcription of UPR genes. Parallelly, ERAD of misfolded proteins is enhanced: this mechanism involves the retrograde translocation of proteins out of the ER and their subsequent degradation by cytosolic 26s proteasomes. The activation of all these pathways results in the attenuation of protein synthesis, the induction of ER chaperones to fold newly synthesized polypeptides and the degradation of misfolded proteins. However, if these mechanisms fail to suppress the induced ER stress, apoptotic cell death is triggered [221].

The principal mediator of the ER stress-related apoptosis is the C/EBP homologous protein (CHOP), also known as DNA-damage-inducible-gene 153 (GADD153). Upregulation of CHOP is achieved downstream of the PERK pathway, through ATF4 modulation. CHOP induces cell death by promoting protein synthesis and oxidation in the stressed ER, via GADD34 and ERO1α activation, respectively. Moreover, Bcl-2 expression is suppressed by CHOP, with subsequent disruption of the balance between pro- and anti-apoptotic proteins [222].

Although IRE1 cascade has been characterized as a primarily pro-survival pathway, it has also been implicated in apoptosis following prolonged ER stress. This is mainly achieved through interaction of IRE1 with the tumor necrosis factor receptor associated factor 2 (TRAF2) and subsequent induction of JNK-mediated cell death [222].

Caspase-4 is also a major player in ER stress. This caspase is bound to the ER membrane where it is kept in an inactive state; its activation has been shown to be regulated by either ER-localized Bax and Bak proteins or by calpains, after intracellular Ca^{2+} storage perturbation [220].

It should be noted that ER stress is also often associated with cytoplasmic vacuolization and paraptosis induction [223].

As mentioned above, several natural compounds have been shown to trigger both apoptosis and paraptosis in tumor cells by activating ER stress. In particular, thapsigargin, an irreversible SERCA inhibitor derived from the Mediterranean *umbrelliferous* plant *T. garganica*, and tunicamycin, an inhibitor of N-acetylglucosamine transferase originally isolated from *S. lysosuperificus*, have been investigated in preclinical settings [224,225]. Similarly, polyphenols, organosulfur compounds, terpenoids, saponins and alkaloids have shown promise as ER stress inducers in various tumor types, including PCa [226].

2.2.4. Autophagy

Autophagy is an evolutionarily conserved catabolic process that is used to deliver cytoplasmic material, such as damaged organelles and protein aggregates, to the lysosome for degradation. It is characterized by the formation of double-membrane vesicles, the autophagosomes, that fuse with lysosomes for cytoplasmic cargo recycling. It responds to a variety of cellular stresses, including not only organelle damage and abnormal protein accumulation but also nutrient deprivation and hypoxia [227,228]. In fact, in case of starvation, autophagy is activated to maintain a provision of proteins and other nutrients, thus promoting cell survival [229]; during hypoxia, autophagy is induced to alleviate the oxidative stress caused by low levels of oxygen [230,231].

The mechanisms underlying the autophagic flux are regulated by a series of proteins. The mTOR pathway is associated with cell growth and cancer progression. mTOR consists of two complexes, mTORC1 and mTORC2, each of which displays distinct functions and localization [232-234]. Activated mTORC1 plays a crucial role in the phosphorylation of autophagy-related proteins (ATGs), thus suppressing autophagy. When mTORC1 is inhibited, such as in case of energy depletion or organelle damage, autophagy is stimulated. In particular, after mTORC1 inhibition, the Unc-51-like autophagy-activating kinase (ULK) complex is activated via dephosphorylation [235]. The activated ULK complex localizes to the phagophore and activates the class III PI3K [236], allowing beclin-1 to recruit different proteins implicated in the autophagosome maturation and elongation [237]. This process is mainly regulated by ATGs. ATG5–

ATG12/ATG16L complexes recruit microtubule-associated protein 1 light chain 3 (LC3), which is converted to the active cytosolic isoform LC3 I by ATG4B and to LC3 II via interactions with phosphatidylethanolamine (PE), ATG3 and ATG7. LC3 II is located in the inner and outer membrane of the autophagosome, enabling it to bind to degraded substrates [238-240]. Mature autophagosomes then fuse with lysosomes to form autolysosomes, which selectively eliminate misfolded proteins and damaged organelles [241] (**fig. 8**).

The p62 protein, also called sequestosome 1 (SQSTM1), is a ubiquitin-binding scaffold protein, which is able to polymerize via an N-terminal PB1 domain and can interact with ubiquitinated proteins via the C-terminal UBA domain. It can also bind directly to LC3 and GABARAP family proteins through a specific sequence motif. The protein is degraded during autophagy and plays a crucial role in linking ubiquitinated proteins to the autophagic machinery to promote their degradation in the lysosome [242].

In cancer biology, autophagy plays dual roles in tumor promotion and suppression. For example, a reduced autophagic flux is unable to successfully degrade damaged organelles or proteins in oxidative-stressed cells, resulting in their malignant transformation. Moreover, BIF-1 proteins, well-known autophagy modulators related to beclin-1, have been found to be mutated or absent in different tumor types, including colorectal and gastric cancer [243-245]. Similarly, mutations of UVRAG proteins, established autophagy regulators, suppress the autophagic flux, leading to increased cell proliferation in colon cancer [246]. On the other hand, a high basal-level of autophagy is observed in several types of RAS-mutated tumors, such as pancreatic cancer [247]. Autophagy inhibition in these tumors suppresses cancer growth and progression [248].

The dual role of autophagy is also reflected in cancer treatment. Several studies have revealed that cancer resistance to chemotherapy can be mediated by autophagy [249,250]. For example, in various cancer cells the efficacy of treatment with 5-fluorouracil (5-FU) is restricted, due to the induction of protective autophagy [251-253]. In addition, in cisplatin-treated ovarian cancer cells autophagy contributes to drug resistance via modulation of the ERK pathway and overexpression of beclin-1 [254,255]. Similarly, cisplatin treatment promotes ATG7-mediated protective

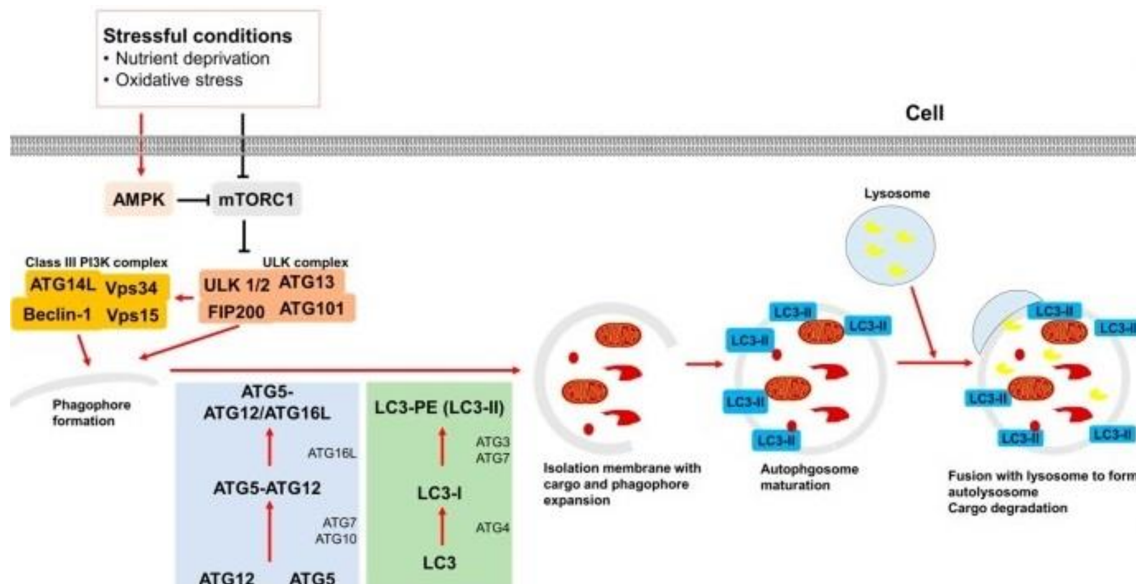

Fig. 8. *The autophagic flux.*

autophagy in esophageal cancer [256,257]. On the contrary, some autophagy regulators, such as rapamycin, rapamycin water-soluble derivatives (everolimus and temsirolimus), chloroquine and hydroxychloroquine have shown promise in cancer therapy. Indeed, everolimus and temsirolimus, which inhibit mTORC1 and induce autophagy, have been approved by FDA for cancer treatment: while everolimus is used to treat breast cancer and progressive neuroendocrine tumors of pancreatic origin (PNET) [258], temsirolimus is used for the treatment of relapsed or refractory mantle-cell lymphoma in the European Union [259-261]. Similarly, preclinical studies have demonstrated that the autophagy inhibitors chloroquine and hydroxychloroquine can suppress bladder cancer and pancreatic adenocarcinoma growth [262,263]. As mentioned above, it should be noted that several nutraceuticals have been found to induce cancer cell death via autophagy [264].

2.2.5. Mitochondrial dysfunction

As reported above, mitochondria play a crucial role in both the apoptotic and paraptotic pathways. Moreover, they can be involved in both ER stress and autophagy.

The ER and mitochondria form a highly dynamic interconnected network implicated in the control of Ca^{2+} homeostasis. Ca^{2+} release from ER is regulated by ryanodine

receptors (RyRs) [265,266], the inositol 1,4,5-triphosphate receptor-gated channels (IP3Rs) and the translocon [267]. As discussed above, restocking of the ER with Ca^{2+} is executed by SERCA [268-270]. Ultimately, the Na+/Ca^{2+} exchanger and Ca^{2+} ATPase located on the plasma membrane participate to Ca^{2+} removal from the cell [271]. Mitochondria take up Ca^{2+} via the outer mitochondrial membrane voltage-dependent anion channel 1 (VDAC1). VDAC1 is highly Ca^{2+}-permeable and modulates Ca^{2+} access to the mitochondrial intermembrane space [272]. Intramitochondrial Ca^{2+} controls energy metabolism by enhancing the rate of NADH production through modulation of critical enzymes in the tricarboxylic acid cycle and fatty acid oxidation. However, once cytoplasmic and intramitochondrial Ca^{2+} rises above a certain threshold, as frequently observed in case of ER stress, the voltage- and Ca^{2+}-dependent high-conductance channel in the inner membrane, known as the mitochondrial permeability transition pore (mPTP), opens, leading to cell death by apoptosis [273,274]. Similarly, cell Ca^{2+} overload has been associated with mitochondrial swelling and paraptotic cell death [223].

The selective degradation of mitochondria by autophagy is called mitophagy. It often occurs to eliminate stressed or damaged mitochondria. There are several pathways regulating mitophagy: so far, the PTEN-induced kinase 1 (PINK1)/Parkin pathway is the best characterized. PINK1 is a 64-kDa protein displaying a mitochondrial targeting sequence (MTS), which allows its recruitment to the mitochondria. In healthy organelles, it is imported through the outer membrane via the TOM complex and partially through the inner mitochondrial membrane via the TIM complex. The process leads to the protein cleavage into a 52-kDa fragment, which is then degraded by the mitochondrial proteases; this keeps the concentration of PINK1 in check in healthy mitochondria [275]. In stressed or damaged mitochondria, the inner mitochondrial membrane depolarizes. Since this membrane potential is necessary for the TIM-mediated protein import, PINK1 is no longer transferred into the inner membrane in unhealthy organelles, and its concentration increases in the outer mitochondrial membrane. PINK1 can then recruit the cytosolic E3 ubiquitin ligase Parkin and activate it via phosphorylation at S65 [276-278]. Parkin ubiquitylates proteins in the outer mitochondrial membrane [279],

particularly mitofusins 1 and 2 (Mfn1 and 2) [277]. The ubiquitylation of mitochondrial surface proteins brings in mitophagy initiating factors. In particular, Parkin promotes ubiquitin chain linkages on both K63 and K48. K48 ubiquitination initiates protein degradation, leading to passive mitochondrial degradation. K63 ubiquitination is thought to recruit the autophagy mediator LC3, which induces mitophagy.

It should be noted that mitochondrial impairment often leads to metabolic dysfunction and redox homeostasis alteration in the cell. Indeed, mitochondria play a key role in the regulation of bioenergetic metabolism by generating ATP through electron transport and oxidative phosphorylation (OXPHOS) in conjunction with the oxidation of metabolites by tricarboxylic acid (TCA) cycle and catabolism of fatty acids by β-oxidation. In addition, they are involved in the control of ROS production [280-284]. In this regard, ROS generation is often increased in cancer cells. This makes them more susceptible to further redox status alterations. Indeed, in cancer, ROS overproduction is known to be involved in both apoptosis and paraptosis, as well as in mTOR-mediated autophagy [285-288].

Mitochondria can not only undergo swelling and autophagic degradation, but also fragmentation. In fact, they constantly undergo fusion and fission [289].

Mitochondrial fusion is regulated by the pro-fusion proteins Mfn 1 and 2 and optic atrophy 1 (OPA1). Mfn 1 and 2 are large GTPases located on the outer mitochondrial membrane, while OPA1 is a dynamin-related GTPase that plays a crucial role in inner membrane fusion. Eight OPA1 isoforms exist, and they are obtained by alternative splicing and alternative processing at two cleavage sites located between the N-terminal transmembrane domain and the first heptad repeat. In normal conditions, these isoforms are constitutively cleaved by the intermembrane space AAA protease Yme1, so that the short and long forms of OPA1 (S- and L-OPA1) are generated [290]. In case of mitochondrial impairment, such as after treatment with the mitochondrial uncoupler CCCP, L-OPA1 is further cleaved by an inducible metalloprotease called OMA1. This cleavage leads to mitochondrial fragmentation by preventing mitochondrial fusion [291,292].

Similar to mitochondrial fusion, mitochondrial fission requires a dynamin-related GTPase known as Drp1. Drp1 is a cytosolic protein, but it can be recruited to the surface of mitochondria to trigger mitochondrial fission [293,289].

2.3. Tocotrienols and cancer

Vitamin E was discovered by Herbert Evans and Katharine Bishop in 1922 [294] and isolated by Evans and Gladys Emerson in 1935 [295]. Its hydrophobic components are divided into two groups: α-, β-, γ- and δ-tocopherols (TPs) and α-, β-, γ- and δ-tocotrienols (TTs). The critical chemical structural difference between them is that the chromanol ring, common to both groups, is linked to a saturated isoprenoid side chain in TPs and to an unsaturated isoprenoid side chain in TTs. Moreover, each isoform of both TPs and TTs differs from others in the number and position of methyl groups on the chromanol ring: the α and β isomers are trimethylated, the γ isomers are dimethylated and the δ isomers are monomethylated (**fig. 9**).

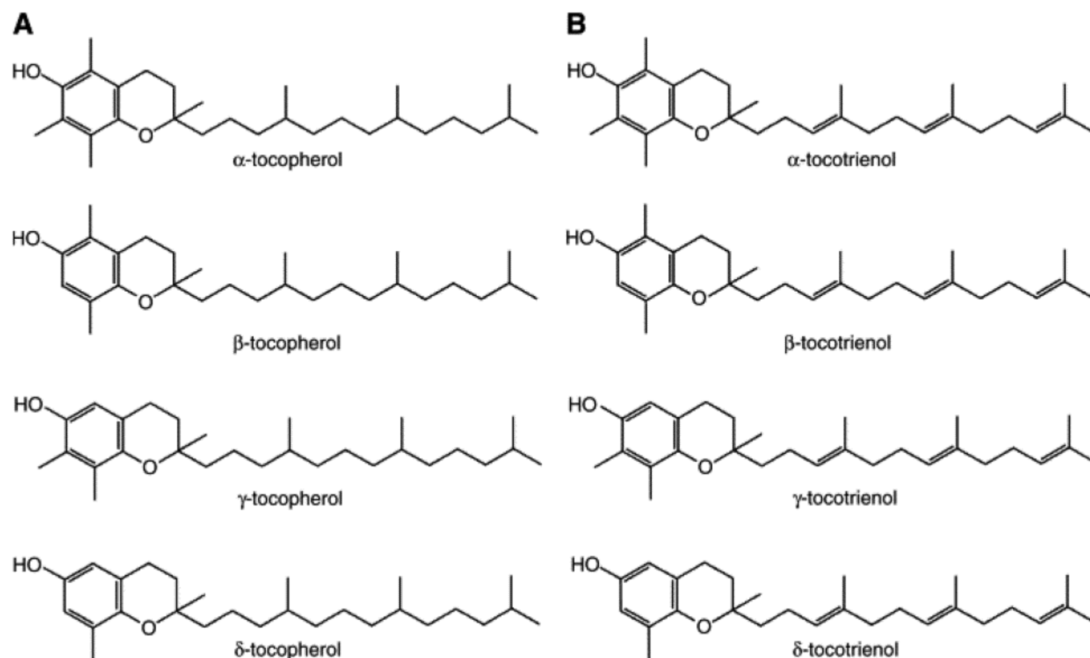

***Fig. 9**. Chemical structure of vitamin E derivatives. **A**. Tocopherols; **B**. Tocotrienols.*

The metabolism of TTs is still not clear. They are absorbed in the small intestine in the presence of bile salts and transported to body tissues through the blood after α-TP transport protein-mediated sequestration into liver lipoproteins. However, TTs seem to have lower affinity than TPs for α-TP transport protein, and various studies have pointed out that TPs can interfere with TTs benefits by lowering their intestinal absorption and increasing their catabolism in the liver [296,297]. Despite these observations, preclinical trials have reported high activity of these compounds after oral administration, without significant side effects and toxicity [298,299]. High bioavailability and safety of TTs were also demonstrated in healthy human subjects and patients with breast and pancreatic cancer [300-302].

In nature, TTs are present in many plants, cereals, seeds, nuts and grains, as well as in the oils derived from them. They are particularly abundant in palm oil, which represents the main source of TTs and in particular of γ-TT [303], annatto (*Bixa orellana* L.) seeds, which contain about 150 mg δ-TT/100 g dry seeds with no TPs [304], and rice bran, containing high levels of α- and γ-TT [305,306]. Other sources of TTs are wheat germ, grapefruit, hazelnuts, olive oil, sunflower oil and flaxseed oil [307,308].

In recent years, TTs have emerged as one of the most effective class of natural compounds for preventing and ameliorating cardiovascular and neurodegenerative diseases, as well as hyperlipidemia, inflammation, diabetes and osteoporosis [309-312]. In addition, they have demonstrated promising potential as anti-cancer agents (**fig. 10**).

2.3.1. Tocotrienols in prostate cancer

TTs were reported to exert anti-proliferative and pro-apoptotic effects in different PCa cell lines by targeting several signaling pathways, such as PI3K/AKT/mTOR, STAT, TFGβ receptor and NF-κB cascades, as well as cyclins and the cell cycle inhibitors p27 and p21 [313-317].

It is known that cancer stem cells (CSCs) represent only a small subpopulation of cancer cells within a tumor mass. However, their self-renewal ability and their capacity to differentiate into the entire heterogeneous tumor cell bulk make them important therapeutic targets. Moreover, they appear to be implicated in the resistance to

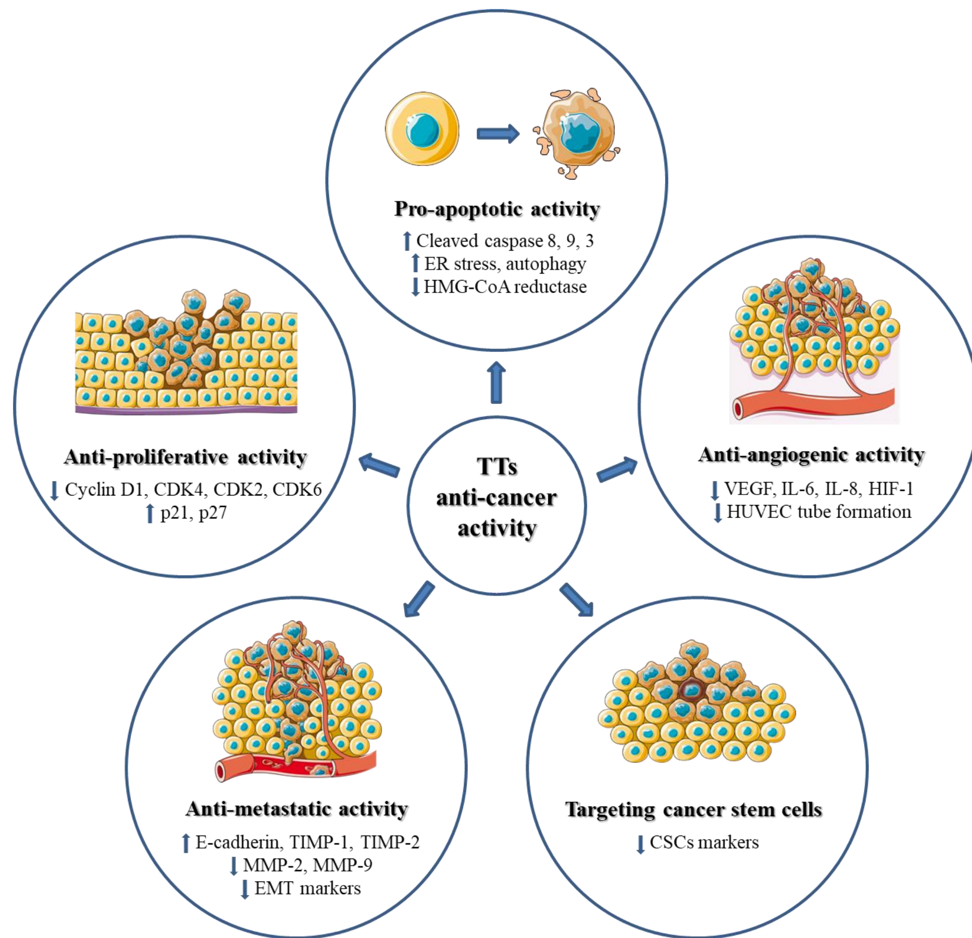

Fig. 10. *Anti-cancer effects of tocotrienols.*

standard cancer treatments, also promoting tumor recurrence and metastasis [318]. It has been shown that γ-TT significantly decreases the expression of CD133 and CD44 CSC markers in PC3 and DU145 castration-resistant PCa cells, also suppressing their anchorage-independent growth and spheroidogenic ability. Moreover, γ-TT pretreatment of PCa cells resulted in the inhibition of tumor initiation after their inoculation in nude mice. Finally, despite the high resistance of CD133-positive PC3 cells to docetaxel treatment, they were as sensitive to γ-TT as the CD133-depleted population [319]. Similar studies were performed by Lee *et al.*, who confirmed the γ-TT ability to specifically target the CSC subpopulation in different PCa cell lines and mouse models, leading to a significant suppression of the proliferation of the castration-resistant tumors [320].

Recent evidence suggests that also δ-TT can inhibit the proliferation of prostate CSCs under hypoxic conditions, by specifically inactivating the HIF-1α cascade [321].

In PCa, TTs were reported to potentiate the anti-tumor activity of lovastatin *in vitro* [322]. A combination treatment with δ-TT and geranylgeraniol was also shown to synergistically suppress the viability of DU145 PCa cells, inducing a significant downregulation of the expression of HMG-CoA reductase, as well as an interesting reduction in membrane K-RAS protein levels [323].

2.1.1. Tocotrienols in other tumors

Tocotrienols in breast cancer

γ-TT was found to exert both anti-proliferative and pro-apoptotic activity in breast cancer cells. In particular, it was shown to reduce the levels of cyclin D1 and cyclin-dependent kinases (CDK) 2, 4 and 6 and to increase the expression of CDK inhibitors [324,325]; to suppress both the PI3K/Akt/mTOR and the Ras/Raf/MEK/ERK signaling pathways and to decrease c-Myc levels [326]; to induce intrinsic apoptosis accompanied by cytochrome *c* release, mitochondrial membrane depolarization, caspase activation, DNA fragmentation and poly(ADP-ribose) polymerase (PARP) cleavage [327,328]; to trigger the extrinsic apoptotic pathway by activation of caspase-8 [329].

δ-TT was also demonstrated to exert potent anti-tumor effects in mammary cancer cells, by reducing proliferation through downregulation of the HMG-CoA reductase activity and inhibition of cholesterol synthesis [330] and by inducing oxidative stress-related mitochondrial apoptosis [331].

The HER-2 receptor normally promotes breast cell proliferation. Amplification of this oncogene occurs in 30% of breast tumors, thus representing an important biomarker and target for therapy [332]. Alawin *et al.* demonstrated that HER-2 receptors and TTs specifically accumulate in breast cancer cell lipid raft microdomains. Moreover, they found that TTs profoundly alter the composition of the lipid rafts, with subsequent disruption of their integrity and inactivation (due to reduced dimerization and phosphorylation) of the associated HER-2 receptors and of the downstream signaling pathways [333].

Almost 70% of human breast cancers are estrogen-dependent and estrogen receptor-positive. TTs were shown to promote the nuclear translocation of the anti-proliferative Estrogen Receptor (ER) β and to decrease the tumorigenic ERα expression [334]. On the other hand, Khallouki *et al.* suggested that δ-TT can induce cytotoxic effects in breast cancer cells independently of their ER status [330].

As illustrated above, different natural compounds were reported to induce ER stress- and autophagy-mediated death in cancer cells: among them, γ-TT specifically triggered both of these pro-apoptotic pathways in breast cancer cells [335-339].

In breast cancer, TTs exhibited both anti-metastatic and anti-angiogenic properties, associated with the inhibition of Met/hepatocyte growth factor receptor and Rac1/WAVE2 cascade and the downregulation of VEGF expression, respectively [340-343].

TTs were shown to specifically eliminate the breast CSCs subpopulation, alone or in combination with simvastatin [344,345].

In +SA mammary tumor cells TTs treatment synergistically increased the anti-cancer activity of synthetic drugs, such as tyrosine kinase inhibitors (erlotinib and gefitinib), statins (simvastatin, mevastatin and lovastatin) and the COX-2 selective nonsteroidal anti-inflammatory agent celecoxib, through suppression of HERB2-4 receptors expression levels and inhibition of AKT and MAPK pathways [346-348]. Furthermore, in multidrug-resistant MCF-7/ADR cells γ-TT significantly reduced the expression of P-gp, leading to enhanced accumulation of doxorubicin in cells and subsequent G2/M cell cycle arrest and apoptosis [349]. Similar synergistic effects were shown by TTs when given in combination with several natural compounds: in murine malignant mammary epithelial cells, the co-treatment with sesamin, a lignan contained in sesame seeds and oil, not only improved TTs bioavailability by reducing their metabolic degradation but also exhibited a synergistic inhibitory effect on the EGF-dependent proliferative pathway [350-352]; the addition of a polyphenol, such EGCG or resveratrol, potentiated the γ-TT-induced downregulation of cyclin D1 and Bcl-2 expression in MCF-7 human breast cancer cells, and the triple combination of these compounds synergistically up-regulated the expression of NAD(P)H Quinone Dehydrogenase 1 (NQO1), an enzyme

activated in case of redox imbalance [353]; a combination of TTs and oridonin caused a significant additive effect in decreasing +SA cell viability through suppression of AKT/mTOR signaling and elevation of apoptosis (caspase-3 and Bax/Bcl-2 ratio) and autophagy (Atg and Beclin-1) markers [354].

It should be noted that also semisynthetic redox-silent TT oxazine derivatives were found to successfully inhibit breast tumor growth, both *in vitro* and *in vivo* [355-357]. In particular, they successfully counteracted the $CoCl_2$-induced increase of HIF-1α levels, with a parallel inactivation of the AKT/mTOR pathway and of its downstream targets p70S6K and eIF-4E1. In addition, TT oxazine derivative treatment resulted in a blockade of the $CoCl_2$-mediated VEGF overexpression.

A five-year double-blinded and placebo-controlled clinical trial was conducted in 250 women with early breast cancer to investigate the TTs adjuvant potentials when given in combination with tamoxifen [358]. The patients, 40-60 years old, with either stage I or II estrogen receptor-positive breast cancer, were non-randomly assigned to two groups: the treatment group was administered 400mg/day TRF plus tamoxifen while the control group was given placebo plus tamoxifen. The 5-year breast cancer-specific survival was 98.3% in the treatment group and 95% in the control group, while the 5-year disease-free survival was 86.7% and 83.3%, respectively. The mortality risk was 60% lower in the TRF group versus controls but it was not statistically significant, probably as a result of the small sample size of the experiment.

Tocotrienols in cervical cancer

TTs were reported to inhibit HeLa cervical cancer cell proliferation through downregulation of the expression of cell cycle-related proteins, such as cyclin D3, p16 and CDK6. Moreover, the induction of HeLa cell death by TTs appeared to be associated with the upregulation of Interleukin-6 (IL-6) [359].

Comitato *et al.* demonstrated that γ- and δ-TT induce apoptosis in cervical cancer cells, by triggering molecular signals associated with ER stress, such as IRE-1α phosphorylation, XBP-1 alternative splicing and CHOP enhanced transcription [360]. Furthermore, they observed significant Ca^{2+} release from the ER membranes to the

cytoplasm, as well as an interesting modulation of isoprenoid, sterol and steroid biosynthesis and metabolism, with SCD, LPIN and SREBPF1-2 downregulation.

γ-TT was found to specifically target Src homology 2 domain-containing phosphatase 2 (SHP2) and the RAS/ERK signaling pathway in spheres from cervical cancer, inhibiting the CSC subpopulation growth [361].

Tocotrienols in colon cancer

In RKO human colon cancer cells, a TRF preparation triggered intrinsic apoptosis correlated with p53 and caspases activation, Bax/Bcl-2 ratio modulation, chromatin condensation, DNA fragmentation and cell membrane shrinkage [362]. Moreover, γ-TT was reported to profoundly alter sphingolipid metabolism in HCT-116 cells through suppression of dihydroceramide desaturase activity and activation of sphingomyelin hydrolysis, ultimately leading to autophagy and apoptosis [363].

It has been recently demonstrated that TTs can trigger paraptosis in SW620 and HCT-8 human colon carcinoma cells through inhibition of the Wnt signaling pathway and downregulation of c-jun, cyclin D1 and β-catenin levels [364,365].

A synergistic antitumor activity of γ-TT and different synthetic and natural anti-cancer agents was observed: the co-treatment with capecitabine synergistically decreased Ki-67, cyclin D1, NF-κB, CXCR4 and MMP-9 expression levels in a nude mouse xenograft model of human colorectal cancer [366]; addition of atorvastatin to tocotrienol treatment enhanced the disruption of RhoA signal transduction in HT29 and HCT116 human colon cancer cells, and a triple combination with celecoxib resulted in a synergistic induction of G0/G1 phase cell cycle arrest and apoptosis [367]; 6-gingerol, the bioactive constituent of ginger, potentiated the γ-TT pro-apoptotic activity in HT29 and SW837 human colorectal cancer cell lines, inducing caspase-3 activation and significant morphological changes, such as cell shrinkage and pyknosis [368].

δ-TT was shown to suppress hypoxia-induced VEGF, IL-8 and COX-2 synthesis in DLD-1 human colorectal adenocarcinoma cells [369], as well as to inhibit tube formation, migration and adhesion of HUVEC cells grown in DLD-1 conditioned medium [370]. These results were also confirmed by *in vivo* experiments [370,371].

Rice bran is not only enriched in δ-TT but also in ferulic acid. Eitsuka *et al.* demonstrated that co-treatment with both these natural compounds significantly increased the intracellular concentration and anti-tumor activity of δ-TT in DLD-1 cells, suggesting that ferulic acid improves the bioavailability as well as the therapeutic effectiveness of δ-TT [372]. In particular, this combination treatment was shown to synergistically down-regulate the expression of Human Telomerase Reverse Transcriptase (hTERT), the catalytic subunit of telomerase, thus suppressing its proliferative activity.

Tocotrienols in gastric cancer

γ-TT was first demonstrated to induce intrinsic apoptosis in human gastric cancer cells through the suppression of the MAPK signaling [373,374].

γ-TT was also found to exert potent anti-metastatic and anti-angiogenic activity in gastric cancer. In particular, it inhibited cell migration and invasion capability, by reducing the expression of the matrix metalloproteinases MMP-2 and MMP-9 and by increasing the levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2 [375], and significantly counteracted the hypoxia-mediated HIF-1α overexpression and VEGF synthesis by modulation of the ERK signaling pathway [376]. Furthermore, it significantly decreased the expression of VEGFR-2 in HUVEC cells grown in a conditioned medium of gastric adenocarcinoma cells [377].

In addition to its pro-apoptotic, anti-metastatic and anti-angiogenic effects, γ-TT was reported to enhance the antitumor activity of capecitabine in human gastric cancer cell lines, as well as in nude mice xenografted with human gastric cancer cells [378].

Tocotrienols in lung cancer

MicroRNAs (miRNAs) are endogenous, ~22 nucleotides, non-coding RNAs that play key regulatory roles in animals and plants by inducing transcriptional silencing through mRNAs cleavage or translational arrest. miRNAs may function as either oncogenes or tumor suppressors (oncomirs), depending on the specific cancer type [379]. Ji *et al.* observed that δ-TT could inhibit nonsmall cancer cell growth and invasiveness through upregulation of miR-34a, which resulted in decreased expression of Notch-1 and its

downstream targets, such as Hes-1, cyclin D1, survivin and Bcl-2 [380]. Moreover, they found that δ-TT exhibited a significant synergistic anti-cancer effect when given in combination with cisplatin. This was related to a reduction of the NF-κB DNA binding activity and to an increase in cleaved caspase-3 and PARP expression [381].

TTs were also shown to potentiate lovastatin-mediated cell growth arrest in the A549 human lung carcinoma cell line [382].

Tocotrienols in pancreatic cancer

Anti-proliferative and pro-apoptotic effects of δ-TT, mediated by $p27^{Kip1}$-dependent cell cycle arrest [383], inhibition of HMG-CoA reductase activity [384] or HER2 [385] and EGR-1/Bax pathway activation [386], were shown in pancreatic cancer cells. Similar results were obtained after γ-TT treatment [387].

TTs significantly suppressed the invasive behavior through downregulation of specific epithelial-mesenchymal transition (EMT) biomarkers (such as N-cadherin, vimentin and MMP9) in L3.6pl and Mia PaCa-2 cells, both *in vitro* and *in vivo* [388].

δ-TT successfully targeted and eliminated pancreatic ductal adenocarcinoma (PDAC) stem-like cells, decreasing the expression of CSC self-renewal-promoting transcription factors, Oct4 and Sox2, and delaying tumor onset in mice [388].

Promising results were reported in pancreatic cancer cells treated with polyethylene glycol (350 and 1000) succinate derivatives of TTs [389]. In addition, *in vitro* studies pointed out that entrapment of gemcitabine/γ-tocotrienol or paclitaxel/γ-tocotrienol lipid conjugates into nanoemulsions significantly enhanced their anti-tumor effects when compared to the free drug [390,391].

Springett *et al.* investigated δ-TT efficacy and safety in patients with PDAC [302]. In this phase I preoperative clinical trial, 25 patients were given crescent oral doses of δ-TT (from 200 to 3200 mg) daily for 13 days before surgery and one dose on the day of surgery. Except for one case of drug-related grade 1 diarrhea registered at the higher daily dose level, the treatment was well tolerated, with no dose-limiting toxicity. δ-TT exhibited an effective half-life of about 4 hours, rapidly reaching bioactive levels in blood and inducing apoptosis-associated caspase-3 cleavage in cancer cells. In particular, the most effective δ-TT dose was between 400 and 1600 mg.

Tocotrienols in skin cancer

We recently demonstrated that δ-TT induces ER-stress-mediated apoptosis in human melanoma cells *in vitro* and in tumors *in vivo*, through the activation of the PERK/p-eIF2α/ATF4/CHOP, IRE1α and caspase-4 ER stress-related branches [392]. Moreover, we observed that, unlike vemurafenib, it can selectively target and eliminate the melanoma ABCG2-positive CSC subpopulation, successfully inducing disaggregation of A375 melanospheres and reducing the spheroid formation ability of sphere-derived cells [393].

γ-TT also exhibited apoptosis-inducing and invasion-suppressing activity in malignant melanoma cells. In particular, it was reported to inhibit NF-κB, EGF-R and Id family proteins, to activate the JNK signaling pathway, to decrease different mesenchymal markers levels and to restore E-cadherin and γ-catenin expression [394].

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor, which controls many biological and physiological processes in response to aromatic hydrocarbons, such as cellular proliferation and differentiation, tissue development, immune and toxic response and skin barrier homeostasis. Upon ligand binding to AhR, the activated complex translocates to the cell nucleus, where it forms a functional heterodimer with the AhR nuclear translocator (ARNT), with subsequent interaction with DNA and transcriptional activation of several target genes (in particular p21 and Bax) by binding to the xenobiotic responsive element [395]. Yamashita *et al.* demonstrated that γ-TT induces AhR expression in a dose-dependent manner in B16 mouse melanoma cells and enhances its sensitivity to baicalein, a flavone particularly abundant in the roots of *Scutellaria baicalensis* and *Scutellaria lateriflora*, which can inhibit tumor cell growth by acting as a ligand of AhR and thus upregulating p21 and Bax levels [396].

Synergistic antitumor activity of TTs and lovastatin was evidenced in murine B16 melanoma cells, as well as in C57BL6 mice bearing B16 xenografts [382].

Improved anti-proliferative effectiveness against A431 and SCC-4 human keratinocyte cancer cells *in vitro* was shown by a hybrid-nanoemulsified TTs delivery system and it was associated with better yield in physicochemical parameters, as well

as stability in chemical and structural composition [397]. Interestingly, an orally administered γ-TT nano-formulation also exhibited enhanced radioprotection compared to γ-TT alone in CD2F1 mice exposed to total body γ radiation [398].

Transferrin receptors are frequently expressed in tumor cells, thus representing a potential target for the delivery of anti-cancer drugs into the tumor mass. Karim *et al.* reported that α-TT entrapped in transferrin-bearing multilamellar vesicles possesses potent growth-suppressing activity in A431 human epidermoid carcinoma cancer cells and B16-F10 murine melanoma cells. Moreover, the intravenous administration of these vesicles to mice bearing A431 and B16-F10 tumors successfully inhibited cancer progression, without visible side effects [399].

AIMS

PCa is the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths among men in Western countries [400]. This pathology initially responds to androgen deprivation therapy, but often progresses into CRPC, a condition where cancer cells acquire the ability to escape cell death and become resistant to current therapies, particularly to docetaxel-based chemotherapy [401]. Thus, new therapeutic approaches for this disease are urgently needed.

In the last decade, the interest in natural compounds has increased, because of their anti-tumor properties and their lesser toxicity with respect to standard therapies. TTs, vitamin E components, were reported to exert significant anti-cancer activities in different tumors. Thus, the aim of this work was to assess the effects of δ-tocotrienol (δ-TT) on the viability of human CRPC cell lines (PC3 and DU145), together with the molecular mechanisms associated with its activity.

Apoptosis is commonly induced by natural products, including TTs, in a variety of tumors [316,327-329,360,373,374,384-387,392]. Moreover, δ-TT has been recently demonstrated to trigger paraptosis, a non-conventional type of programmed cell death, in human colon cancer cells [363-364]. For these reasons, the first task of this project was to assess the role of apoptotic and paraptotic cell death in the δ-TT-mediated cytotoxicity in CRPC cells.

ER stress and autophagy are implicated in the pathogenesis of various diseases, including cancer [402, 403]. Emerging evidence indicates that pharmacological targeting of these two molecular pathways may represent an effective therapeutic strategy to treat tumors [402,404]. Different natural compounds have been shown to induce ER stress- and autophagy-mediated cell death in cancer [220, 405]. As second task of this project, we investigated the involvement of ER stress and autophagy in the δ-TT anti-tumor activity in CRPC.

Mitochondria play a critical role in the generation of metabolic energy in eukaryotic cells, and they are also involved in the regulation of redox homeostasis. In addition, they are known to be implicated in both apoptosis and paraptosis, as well as in ER stress and autophagy. The last task of this project was then focused on the clarification of the molecular mechanisms underlying the pro-death pathways activated in δ-TT-treated

CRPC cells, with special regard to mitochondrial functional and structural impairment (*i.e.*, metabolic dysfunction, mitochondrial dynamics, Ca^{2+} overload and ROS production).

MATERIALS AND METHODS

Chemicals

δ-TT was purified from a commercial extract of Annatto seeds (*Bixa orellana*) (kindly provided by American River Nutrition Inc., Hadley, MA, USA), as previously described [406].

The following primary antibodies were utilized: caspase 3 (9656), cleaved caspase 3 (9664), PARP (9542), BiP (3177), eIF2α (5324), p-eIF2α (3398), ATF4 (11815), CHOP (2895), IRE1α (3294), PDI (3501)AMPK (5832), p-AMPK (5832), OPA1 (80471), MFN2 (9482), Drp1 (14647), JNK (9252), p-JNK (4668), P38 (8690), p-P38 (4511), AKT (2938), p-AKT (9271), mTOR (2983), p-mTOR (5536), PINK (6946) (all from Cell Signaling Technology Inc., Danvers, MA, USA), cytochrome *c* (sc-13560) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) LC3 (L8918) and α-tubulin (T6199) (both from Sigma-Aldrich, Milano, Italy), p62/SQSTM1 (PA5-20839) (from Thermo Fisher Scientific, Rodano, Milano, Italy), Total OXPHOS (ab110411) (from Abcam, Cambridge, UK). Most of the antibodies were utilized at the concentration 1:1000; cleaved caspase 3 at 1:500 and LC3 at 1:2500.

Horseradish-peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents were from Cyanagen (Bologna, Italy).

Alexa Fluor 488 and 594 secondary antibodies were from Thermo Fisher Scientific.

Z-VAD-FMK (the pan-caspase inhibitor; FMK001) was from R&D System Inc (Minneapolis, MN). The ER stress inhibitors salubrinal (Sal) and 4-PBA (4-phenylbutyrate), the autophagy inhibitors CQ (chloroquine) and Baf (bafilomycin), the translation inhibitor cycloheximide, and analytical grade solvents were from Sigma-Aldrich; 3-MA (3-methyladenine) was from Selleckchem (Munich, Germany). DIDS (disodium 4,4’-diisothiocyanostilbene-2,2’-disulfonate), the blocker of the mitochondrial Ca^{2+} overload, NAC (N-acetyl-L-cysteine), the known ROS scavenger, and dichlorofluorescin (2',7'-dichlorofluorescin diacetate, DCFDA), the oxidant-sensing probe for the detection of ROS levels, were from Sigma-Aldrich.

Cell lines and cell culture

Normal prostate epithelial RWPE-1 (provided by Dr N. Zaffaroni; IRCCS, National Institute of Cancer, Milano, Italy) and cancer (DU145 and PC3) cell lines were from American Type Culture Collection (ATCC, Manassas, VA, USA). RWPE-1 cells were cultured in keratinocyte-SFM medium supplemented with Bovine Pituitary Extracts and EGF (2.5 µM) (Thermo Fisher Scientific), DU145 and PC3 cells in RPMI medium supplemented with FBS (7.5% and 5% respectively), glutamine and antibiotics. Cells were cultured in humidified atmosphere of 5% CO_2/95% air at 37°C. Original stocks of cells were stored frozen in liquid nitrogen; after resuscitation, cells were kept in culture for no more than 10-12 weeks. Cells were detached through trypsin-EDTA solution and passaged once/week. Cell lines were recently authenticated by Short Tandem Repeat (STR) analysis as described in ANSI Standard (ASN-0002) by ATCC Standards Development Organization (SDO).

MTT viability assay

Cells were seeded at a density of 3×10^4 cells/well in 24-well plates for 24 hours and then exposed to the specific compounds. The medium was then changed with MTT solution (0.5 mg/ml) in RPMI without phenol red and FBS; cells were incubated at 37 °C for 15-45 min and violet precipitate was dissolved with isopropanol. Absorbance at 550 nm was measured through an EnSpire Multimode Plate reader (PerkinElmer, Milano, Italy).

Trypan blue exclusion assay

Cells were plated (5×10^4 cells/dish) in 6-cm dishes. After 48 hours, cells were treated with δ-TT (5-20 µg/mL, 24 hours). Adherent (viable) and floating (dead) cells were harvested, stained with Trypan blue 0.4% (1:1 v/v) and counted by Luna automated cell counter (Logos Biosystems, Annandale, VA, USA).

Colony formation assay

Cells were seeded (100-250 cells/well, depending on the cell type) in 6-well plates. After each treatment, a colony formation assay was performed to assess dimensions

and numbers of colonies. Colonies were fixed with 70% methanol and stained with Crystal Violet 0.15%. Images of stained colonies were captured by a Nikon photo camera.

Western blot assay

Cells were seeded at 5×10^5 cells/dish in 10-cm dishes. After each treatment, cells were lysed in RIPA buffer; protein preparations (15-40 µg) were resolved on SDS-PAGE and transferred to nitrocellulose (or PVDF for the Western blot of LC3) membranes. Membranes were incubated with the specific primary antibodies. Detection was done using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Westar Etac Ultra 2.0, XLS075,0100; Cyanagen Srl). α-Tubulin was utilized as a loading control.

Immunofluorescence assay

Cells were seeded at 3×10^4 cells/well in 24-well plates on polylysine-coated 13-mm coverslips for 48 hours before treatments. After each treatment, cells were fixed and stained with the specific primary antibodies, followed by secondary antibodies. Labelled cells were examined under a Zeiss Axiovert 200 microscope with a 63 × 1.4 objective lens linked to a Coolsnap Es CCD camera (Roper Scientific-Crisel Instruments, Roma, Italy).

Morphological analysis

Cells were seeded at 3 or 4×10^4 cells/dish in 6-cm dishes, respectively, and treated with δ-TT. Cytoplasmic vacuolization was analyzed by light microscopy from different fields under a Zeiss Axiovert 200 microscope with a 20 or 32 × 0.4 objective lens linked to a Coolsnap Es CCD camera (Roper Scientific-Crisel Instruments).

For TEM analysis, cell pellets were fixed overnight in a solution containing 2% of paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). Samples were post-fixed in 1% osmium tetroxide in cacodylate buffer at 0°C for 90 minutes, washed, dehydrated and embedded in Epon-Araldite resin. Ultrathin

sections were cut by a Leica Supernova ultramicrotome (Reichert Ultracut E) and stained with lead citrate. TEM was performed with a Zeiss EM10 electron microscope.

Flow cytometry analysis

After the indicated treatments, flow cytometry analysis was utilized to analyze: mitochondrial activity (using the fluorescent Mitotracker Orange CMTMRos dye; ThermoFisher Scientific); cytosolic and mitochondrial Ca^{2+} levels (after staining the cells with the Fluo-3 AM and Rhod-2 AM fluorescent probes, respectively; Invitrogen Life Technologies, Monza, Italy); intracellular ROS levels (using the fluorescent probe dichlorofluorescin, DCFDA; Invitrogen Life Technologies). The flow cytometry analyses were performed with a Novocyte3000 instrument (ACEA Biosciences, San Diego, CA). Data were analyzed with Novoexpress software.

Oxygen consumption

The analysis of OCR (oxygen consumption rate) on whole cells after δ-TT treatments was performed using a Clark oxygen electrode (DW1 electrode chamber, Hansatech Instruments Ltd, Norfolk, UK), as previously described [407]. DU145 and PC3 cells were rinsed in pre-warmed (37 °C) PBS and suspended in coupled respiration buffer (2% free-fatty acid BSA, 25 mM D-glucose, 1 mM Na-pyruvate, 40 µg/ml digitonin) or electron flow buffer (2% free-fatty acid BSA, 2 mM malate, 10 mM Na-pyruvate, 40 µg/ml digitonin, 4 µM carbonyl cyanide m-chlorophenyl hydrazine, CCCP). Then, samples were transferred to the electrode chamber for the measurement of OCR. After the measurement of basal respiration, uncoupled and maximal respiration were analyzed by adding oligomycin (10 µM) and CCCP (10 µM), respectively.

ATP measurement

The effects of δ-TT treatments on ATP production in PC3 and DU145 cells were investigated using a specific ATP colorimetric/fluorimetric assay kit (GeneTex, Alton Pkwy Irvine, CA, USA). Luminescence was analyzed to quantify ATP production.

Statistical analysis

Statistical analysis was performed with a statistic package (GraphPad Prism5, GraphPad Software San Diego, CA, USA). Data are represented as the mean ± SEM of three-four independent experiments. Differences between groups were assessed by t-test or one-way analysis of variance (ANOVA) followed by Dunnet's or Bonferroni's test. A *P* value < 0.05 was considered statistically significant.

RESULTS

δ-TT reduces cell viability and exerts a cytotoxic effect in prostate cancer cells

DU145 and PC3 or normal RWPE-1 prostate epithelial cells were treated with δ-TT (5-20 μg/mL, 24 hours); cell viability was measured (MTT assay). δ-TT decreased the number of viable CRPC cells in a dose-dependent way being significantly effective at 10-20 μg/mL. The IC50 values were 2.91×10^{-5} M and 3.22×10^{-5} M for DU145 and PC3 cells, respectively. The same treatment affected the growth of RWPE-1 cells only slightly and at the highest dose (20 μg/mL; **Figure 1A**). CRPC cells were treated with δ-TT (5-20 μg/mL, 24 hours), then dying (floating) and living (adherent) cells were harvested, stained with Trypan blue and counted. In both cell lines, δ-TT significantly and dose-dependently decreased the number of viable cells and increased the number of dead cells (**Figure 1B**). To obtain growth curve kinetics beyond 24 hours, CRPC cells were treated with δ-TT (15 μg/mL, 36-72 hours); cell viability was then measured. δ-TT significantly and dose-dependently decreased the number of viable cells at each time point (**Figure 1C**), confirming results reported in **Figure 1A**. The cytotoxic activity of δ-TT was investigated by colony formation assay. CRPC cells were treated with δ-TT (15 μg/mL, 48 hours) and left to grow for 11-12 days in the absence of the treatment, according to the cell line-specific proliferation rate. We analyzed (a) the ability of the cells to form colonies (dimensions of colonies) and (b) the survival of colony-forming cells (number of colonies). We observed that untreated cells grew forming colonies while none of δ- TT-treated cells survived to the treatment; moreover, the ability of the cells to form colonies was prevented by the treatment, supporting a cytotoxic effect of δ-TT (**Figure 1D**).

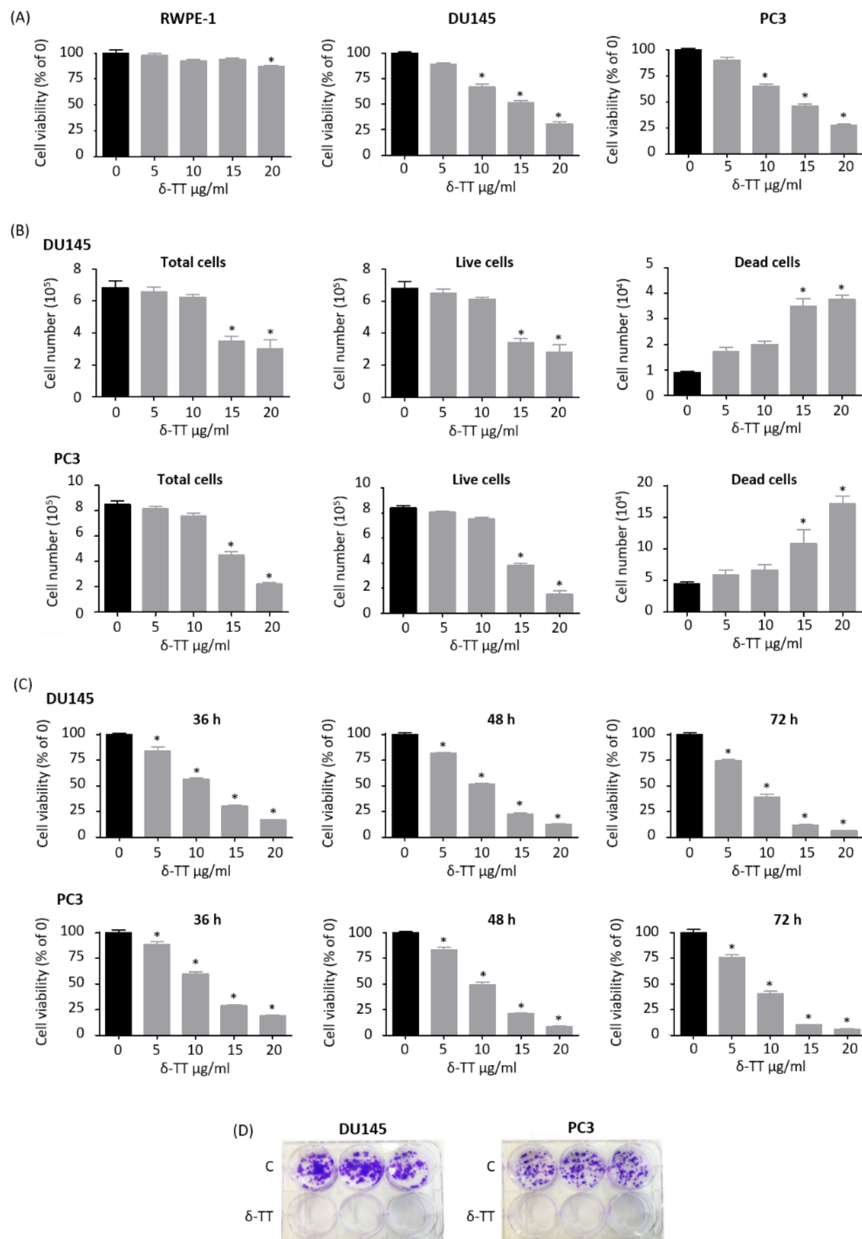

FIGURE 1. δ-TT decreases cell viability and exerts a cytotoxic effect on DU145 and PC3 prostate cancer cells. A, RWPE-1 normal epithelial prostate cells and DU145 and PC3 prostate cancer (PCa) cells were treated with δ-TT (5-20 μg/mL) for 24 h. Cell viability was then evaluated by MTT assay. The IC50 values were 2.91×10^{-5} M and 3.22×10^{-5} M for DU145 and PC3 cells, respectively. **B**, PCa cells were treated with δ-TT (5-20 μg/mL) for 24 h. Total, live and dead cells were evaluated by Trypan blue exclusion assay. **C**, DU145 and PC3 cancer cells were treated with δ-TT (15 μg/mL) for 36-72 h. Cell viability was then evaluated by MTT assay. **D**, PCa cells were treated with δ-TT (15 μg/mL) for 48 h and then, after withdrawal of the treatment, were left to grow for 11-12 d, dependently on the cell line-specific proliferation rate. A colony formation assay was performed to evaluate the ability of the cells to form proliferating colonies (dimensions of colonies) and the survival of colony-forming cells (number of colonies). Each experiment was repeated three times. Data in **A-C** represent mean values ± SEM and were analyzed by Dunnet's test after one-way analysis of variance. *$P < 0.05$ vs 0, controls (vehicle).

δ-TT triggers apoptosis in prostate cancer cells

DU145 and PC3 cells were treated with δ-TT (5-20 µg/mL, 24 hours); the levels of active (cleaved) caspase 3 and PARP were increased by δ-TT treatment (15 and 20 µg/mL) in both cell lines (**Figure 2A**, left panels). Moreover, active caspase 3 and PARP levels increased in both cell lines at 18-24 h of treatment (15 µg/mL) (**Figure 2A**, right panels). Immunofluorescence studies were performed to confirm the involvement of intrinsic apoptosis in the activity of δ-TT (15 µg/ mL, 18 hours). It was observed that, after treatment, cytochrome *c* was diffused in the cytosol and no overlapping with mitochondria could be observed demonstrating its release from mitochondria (**Figure 2B**). CRPC cells were treated with Z-VAD-FMK, the pan-caspase inhibitor (50 µM, 4 hours) before the treatment with δ-TT (15 µg/mL, 24 hours). Cell viability was significantly reduced by δ-TT. Z-VAD- FMK, given alone, did not modify cell viability; however, pretreament of both cell lines with Z-VAD-FMK significantly (even if not completely) reverted the anti-tumor effect of δ-TT (**Figure 2C**).

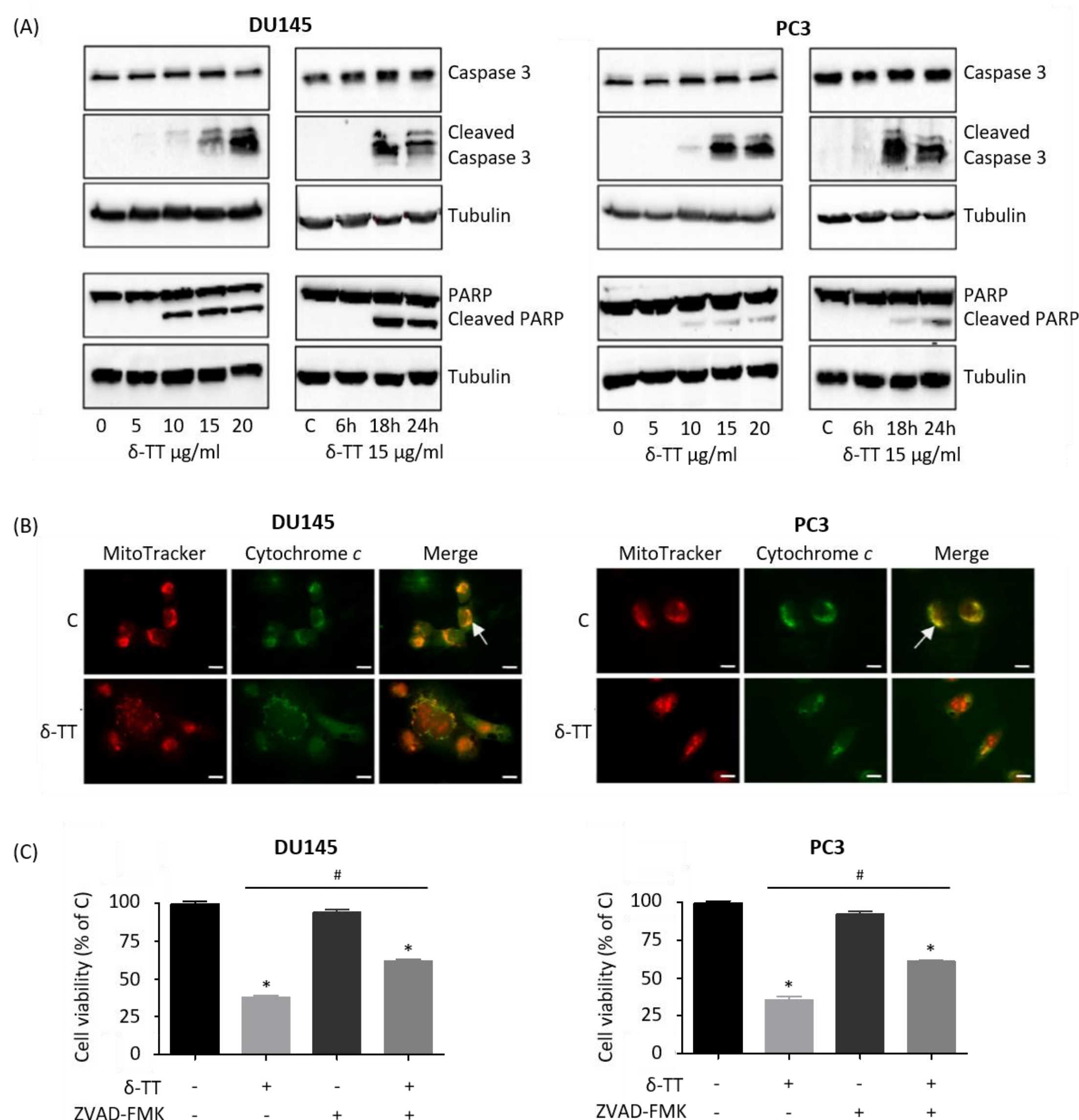

FIGURE 2. δ-TT triggers apoptosis in DU145 and PC3 prostate cancer cells. A, DU145 and PC3 cells were treated with δ-TT (5-20 μg/mL) for 24 h (left panels) or with δ-TT (15 μg/mL) for 6-24 h (right panels). Western blot analysis was carried out to analyze the expression levels of cleaved caspase 3 and PARP. Tubulin expression was evaluated as a loading control. 0 and C, controls (vehicle). One representative of three different experiments, for each of the analyses performed, is shown. **B**, DU145 and PC3 cells were treated with δ-TT (15 μg/mL) for 18 h; the intracellular localization of cytochrome *c* was then evaluated by immunofluorescence analysis. One representative of three experiments performed is shown. Scale bars are 20 μm. The arrow indicates the cytochrome *c*-mitochondrial colocalization in controls cells. **C**, to confirm the involvement of apoptosis in the anti-tumor activity of δ-TT, DU145 and PC3 cells were treated with the pan-caspase inhibitor Z-VAD-FMK (50 μM) for 4 h before the tocotrienol (15 μg/mL for 24 h). Cell viability was then evaluated by MTT assay. Each experiment was repeated three times. Data represent mean values ± SEM and were analyzed by Bonferroni's test after one-way analysis of variance. *P < 0.05 vs controls (vehicle). #P < 0.05 vs δ-TT-treated cells.

δ-TT triggers ER stress in prostate cancer cells

CRPC cells were treated with δ-TT (15 μg/mL, 1-24 hours). The expression of ER stress markers (BiP, eIF2α, p-eIF2α, IRE1α, PDI) and markers of ER stress-related apoptosis (ATF4 and CHOP) were analyzed by Western blotting. δ-TT increased the levels of BiP (18-24 hours) in DU145 and PC3 cells. The levels of peIF2α (but not eIF2α) increased in DU145 (6-18 hours) and PC3 cells (1-24 hours). The expression of IRE1α was increased at 18-24 hours in DU145 and at 1-24 hours in PC3 cells. On the other hand, the levels of the chaperone protein PDI was unaffected in both cell lines. Finally, ATF4 levels were increased at 18-24 hours in DU145 cells and at 6-24 hours in PC3 cells, while those of CHOP increased at 18-24 hours in both CRPC cell lines (**Figure 3A**). The intracellular localization of the transcription factors involved in the ER stress-mediated apoptosis was analyzed in CRPC cells treated with δ-TT (15 μg/mL, 18 hours) by immunofluorescence. In untreated cells, the levels of ATF4 and CHOP were almost undetectable in both cells lines (confirming the results obtained by Western blot). δ-TT treatment triggered the expression of these transcription factors together with their nuclear localization (overlapping staining between TRITC-conjugated antibodies and DAPI; **Figure 3B**). To confirm the specificity of the effects of δ-TT on ER stress-related proteins, CRPC cells were treated with the tocotrienol (15 μg/mL, 24 hours), either in the absence or in the presence of two ER stress inhibitors: salubrinal (Sal, 20 μM) or 4-PBA (2 mM), for 4 and 1 hours, respectively. **Figure 3C** confirms that δ-TT induces the expression of CHOP and ATF4 (as in **Figure 3A**); pretreatment with both ER stress inhibitors significantly reduced the expression of both proteins. These results support that δ-TT triggers ER stress in CRPC cells.

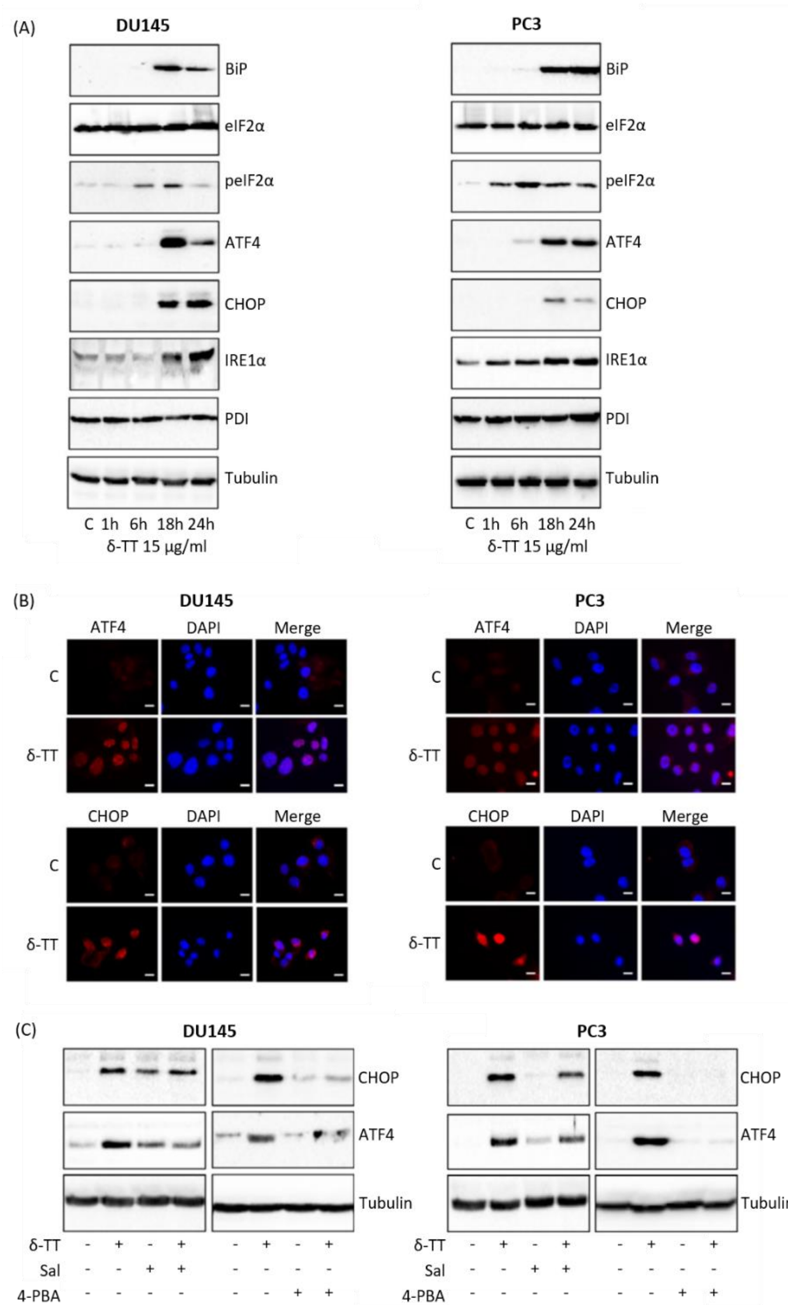

FIGURE 3. δ-TT triggers ER stress in DU145 and PC3 prostate cancer cells. A, DU145 and PC3 cells were treated with δ-TT (15 μg/mL) for 1-24 h. Western blot analysis was performed to investigate the expression levels of ER stress-related proteins (BiP, eIF2α, p- eIF2α, ATF4, CHOP, IRE1α, PDI). Tubulin expression was evaluated as a loading control. **B**, DU145 and PC3 cells were treated with δ-TT (15 μg/mL) for 18 h. The expression levels and intracellular localization of the key transcription factors involved in the ER stress-mediated apoptosis (ATF4 and CHOP) were evaluated by immunofluorescence analysis. C, controls (vehicle). Scale bars are 20 μm. **C**, CRPC cells were pretreated with the ER stress inhibitors salubrinal (Sal; 20 μM) or 4-PBA (2 mM), for 4 and 1 h, respectively, before treatment with δ-TT (15 μg/mL) for 24 h. The effects of the treatments were analyzed on CHOP and ATF expression levels by Western blot. Tubulin expression was evaluated as a loading control. One representative of three different experiments performed is shown.

ER stress mediates the anti-tumor activity of δ-TT in prostate cancer cells

Data from the literature support that ER stress is involved in the anti-tumor activity of δ-TT in cancer cells. To confirm this hypothesis in PCa cells, DU145 and PC3 cells were treated with two ER stress inhibitors: salubrinal (20 μM) or 4-PBA (2 mM), for 4 and 1 hours, respectively, before δ-TT treatment (15 μg/mL, 24 hours). δ-TT markedly increased the expression levels of cleaved caspase 3 and PARP, confirming the results reported in **Figure 2A**. Salubrinal and 4-PBA, given alone, did not modify the expression of these proteins; however, they significantly counteracted the effects of the tocotrienol on the expression of the cleaved forms of both caspase 3 and PARP (**Figure 4A**). **Figure 4B** shows that cell viability was significantly suppressed by δ-TT treatment. Salubrinal and 4-PBA alone did not influence the viability of cancer cells; however, they significantly reverted (although not completely) the cytotoxic activity of δ-TT, in both cell lines. These results demonstrate that ER stress is involved in the anti-cancer activity of δ-TT.

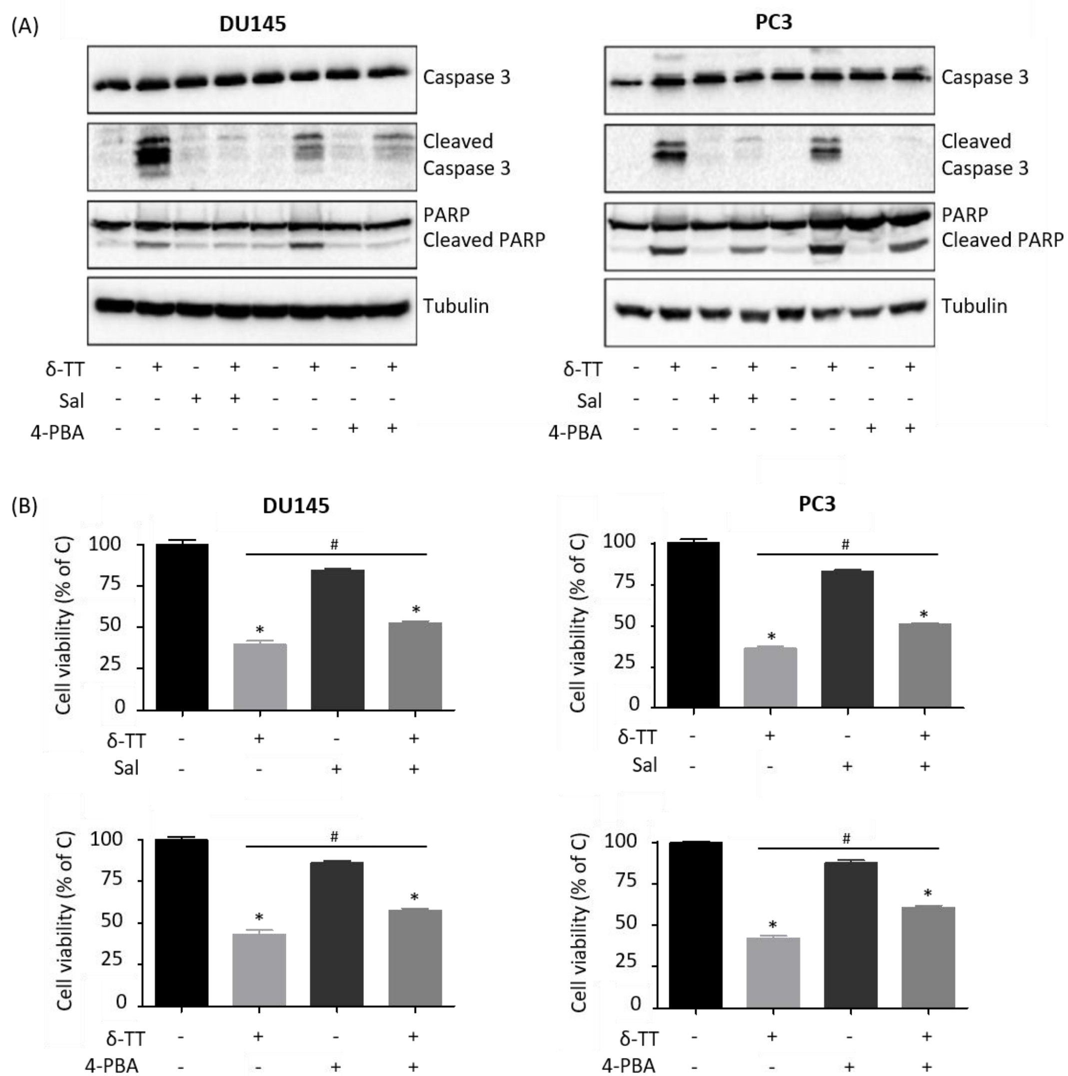

FIGURE 4. ER stress mediates the pro-apoptotic activity of δ-TT in DU145 and PC3 prostate cancer cells. DU145 and PC3 cells were pretreated with the ER stress inhibitors salubrinal (Sal; 20 μM) or 4-PBA (2 mM), for 4 and 1 h, respectively, before treatment with δ-TT (15 μg/mL) for 24 h. **A**, the expression levels of cleaved caspase 3 and PARP were evaluated by Western blot analysis. Tubulin expression was evaluated as a loading control. One representative of three different experiments performed is shown. **B**, cell viability was assessed by MTT assay. Each experiment was repeated three times. Data represent mean values ± SEM and were analyzed by Bonferroni's test after one-way analysis of variance. *$P < 0.05$ vs controls (vehicle). $^{\#}P < 0.05$ vs δ-TT-treated cells.

δ-TT triggers autophagy in PC3 prostate cancer cells

To assess whether δ-TT might trigger the autophagic pathway in CRPC cells, DU145 and PC3 cells were treated with δ-TT (15 μg/ mL, 24 hours). We demonstrated that the tocotrienol markedly increases the expression levels of the autophagy-related proteins LC3-II (increased LC3-II/LC3-I ratio) and SQSTM1/p62 in PC3 cells (at 6-24 and 1-24 hours, respectively) but not in DU145 cells (**Figure 5A**). In line with this observation, by immunofluorescence analysis, we observed that, in basal conditions, LC3 is poorly expressed in both cell lines; δ-TT induced the cytoplasmic accumulation of LC3 (LC3 puncta) and p62 bodies formation in PC3 but not in DU145 cells (**Figure 5B**). These results are in agreement with data reporting that DU145 cells are autophagy-defective due to an alternative splicing of ATG5 transcript and lack of a full-length ATG5 protein [408]. Thus, further studies investigating the involvement of autophagy in δ-TT anti-cancer activity were performed in PC3 cells.

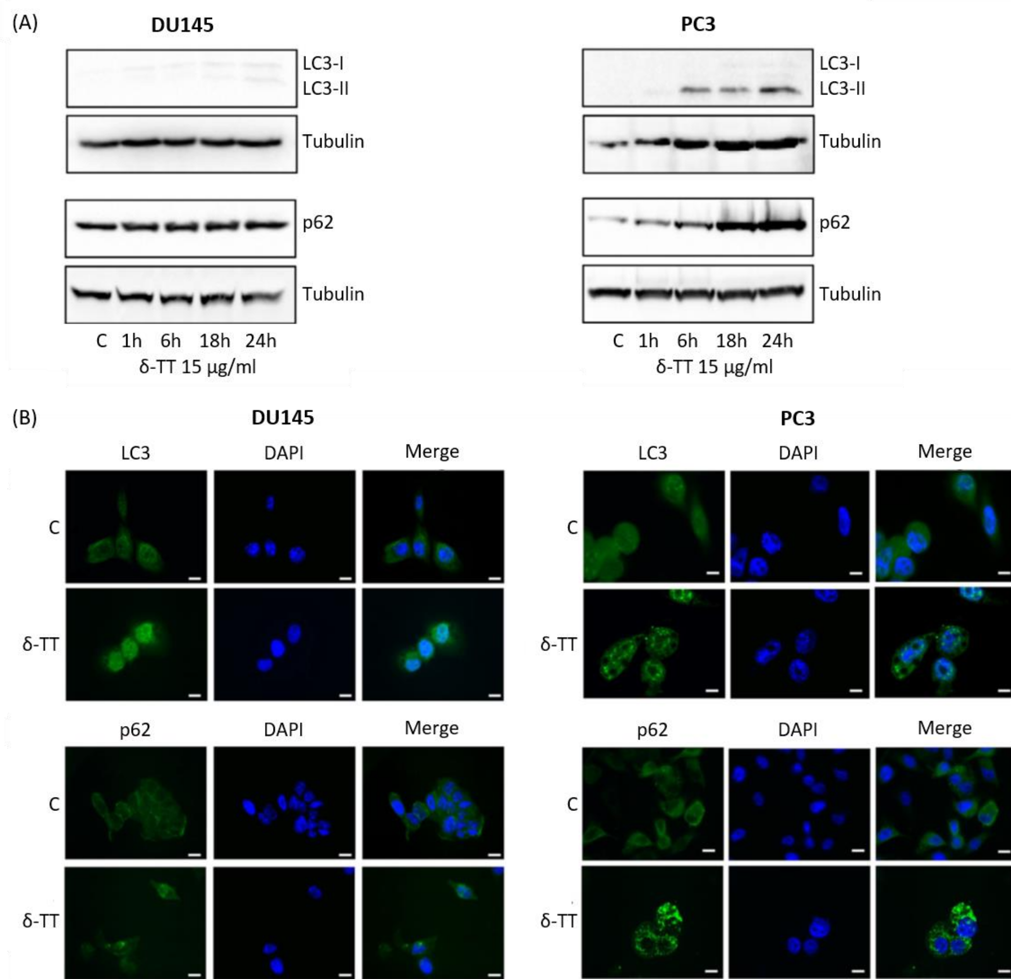

FIGURE 5. δ-TT triggers autophagy in PC3 but not in DU145 prostate cancer cells. DU145 and PC3 cells were treated with δ-TT (15 µg/mL) for 1-24 h. **A**, Western blot analysis was performed to investigate the expression levels of autophagy-related proteins (LC3-II/LC3-I, SQSTM1/p62). Tubulin expression was evaluated as a loading control. **B**, the expression levels and intracellular localization of LC3 and SQSTM1/p62 were evaluated by immunofluorescence. One representative of three different experiments performed is shown. C, controls (vehicle). Scale bars are 20 µm.

δ-TT triggers ER stress-related autophagy in PC3 prostate cancer cells

To confirm the activation of an autophagic flux in PC3 cells, we investigated the presence of autophagosomes in δ-TT-treated (15 µg/ mL, 18 hours) cells by TEM. **Figure 6A** shows that, at variance with control cells (left panel), autophagosomes containing entire organelles surrounded by multilamellar membranes are present in tocotrienol-treated cells (middle panel, boxed area) and localize at the lysosomal level forming autophagolysosomes containing remnants of digested structures (right panel, boxed area). Moreover, cells were pretreated with 3-MA (10 mM), or with CQ (10 µM) or Baf (10 nM) and then with the tocotrienol (15 µg/mL, 24 hours). Pretreatment of the cells with 3-MA (inhibitor of early stage autophagy) inhibited LC3-II expression (decreasing the LC3-II/LC3-I ratio; **Figure 6B**); on the contrary, CQ and Baf (inhibitors of the late phase of autophagy) significantly potentiated the effect of δ-TT on the accumulation of LC3-II (LC3-II/LC3-1 ratio; **Figure 6C**). Similar results were obtained on the expression levels of SQSTM1/62 (**Figure 6B**, **C**). Normally, the activation of autophagy determines a decrease in the expression of SQSTM1/p62, because of its accumulation in autophagosomes and the final degradation into lysosomes. However, SQSTM1/p62 upregulation, and at least transient increases in the amount of this protein, is seen in some situations, such as starvation and ER stress, where there is an increase in its transcription. The results obtained indicate that the levels of SQSTM1/p62 are elevated, but the autophagic flux is not impaired. To assess the involvement of the ER stress in δ-TT-induced autophagy, PC3 cells were pretreated with salubrinal (20 µM, 4 hours) or 4-PBA (2 mM, 1 hours), before treatment with δ-TT (15 µg/mL, 24 hours). We showed that both ER stress inhibitors counteracted the tocotrienol-triggered increase of the LC3-II/LC3-I ratio as well as that of SQSTM1/p62 expression (**Figure 6D**, **E**). In conclusion, in PC3 cells (but not in autophagy-defective DU145 cells), δ-TT-induced autophagy is related to the upstream activation of the ER stress pathways (ER stress-autophagy axis).

Autophagy mediates the anti-tumor activity of δ-TT in PC3 prostate cancer cells

To assess whether autophagy might mediate the pro-apoptotic activity of δ-TT in PC3 cells, cells were pretreated with 3-MA (10 mM, 4 hours) before δ-TT (15 μg/mL, 24 hours). Caspase 3 and PARP cleavage and cell viability were investigated (Western blot and MTT assay). **Figure 6F** shows that δ-TT increased the expression of cleaved caspase 3 and PARP, as previously observed. 3-MA, given alone, did not affect the levels of these proteins; on the other hand, 3-MA significantly counteracted the effects of δ-TT on the expression levels of cleaved caspase 3 and PARP (**Figure 6F**). As expected, cell viability was significantly suppressed by δ-TT, while it was not affected by 3-MA; however, the autophagy inhibitor significantly reverted (although not completely) the cytotoxic effect of δ-TT (**Figure 6G**).

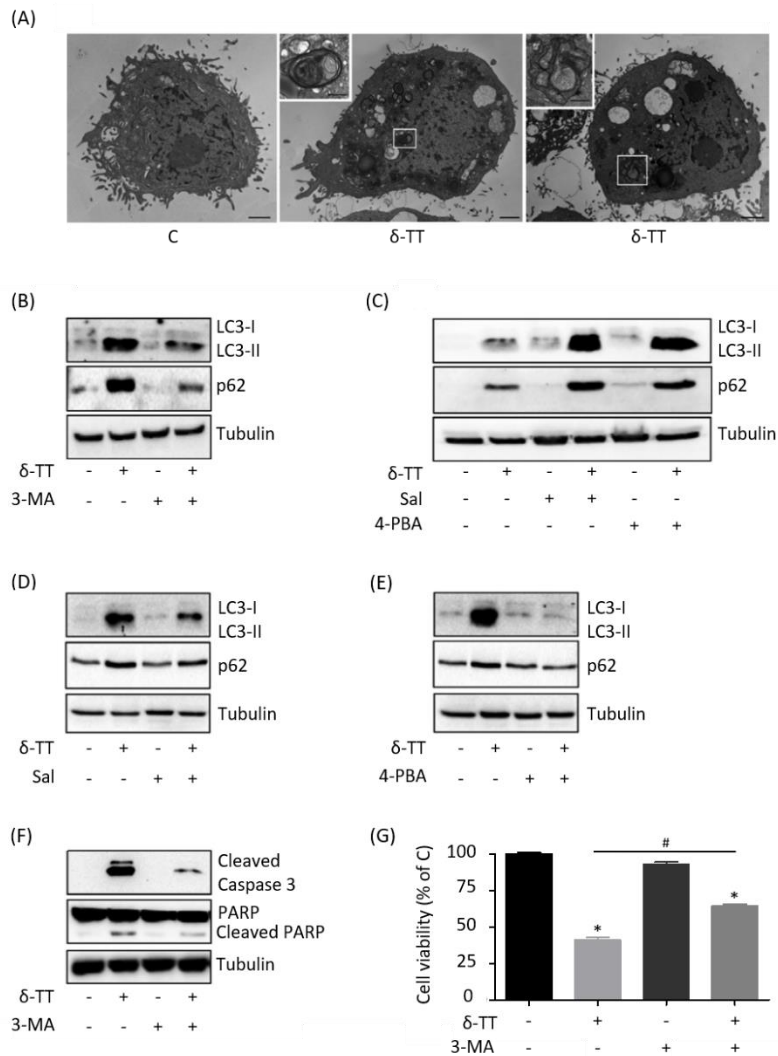

FIGURE 6. Autophagy, related to ER stress, mediates the anti-tumor activity of δ-TT in PC3 prostate cancer cells. A, cells were treated with δ-TT (15 μM) for 18 h and TEM images were selected. Left panel is a TEM image of control cells. Boxed area indicates the presence, in δ-TT-treated cells, of autophagosomes (middle panel) that can localize at the lysosomal level forming autophagolysosomes (right panel). Scale bars are 2 and 0.7 μm for photographs in boxed areas. **B** and **C**, cells were treated with δ-TT (15 μg/mL) for 24 h in the presence of either the inhibitor of early stage autophagy 3-MA (10 mM) (**B**) or the inhibitors of late stage autophagy CQ (10 μM) and Baf (10 nM) (**C**). The LC3-II/LC3-I ratio and SQSTM1/p62 levels were evaluated by Western blot analysis. **D** and **E**, cells were pretreated with the two ER stress inhibitors salubrinal (Sal; 20 μM) for 4 h or 4-PBA (2 mM) for 1 h, and then with δ-TT (15 μg/mL) for 18 h. The LC3-II/LC3-I ratio and SQSTM1/p62 levels were evaluated by Western blot analysis. **F** and **G**, PC3 cells were pretreated with 3-MA (10 mM) for 4 h before tocotrienol treatment (15 μg/mL) for 24 h. The effects of the treatment were analyzed on the expression of apoptosis-related markers, by Western blot (**F**) as well as on cell viability, by MTT assay (**G**). For Western blot analyses, one representative of three different experiments performed is shown. For MTT assay, each experiment was repeated three times and data represent mean values ± SEM and were analyzed by Bonferroni's test after one-way analysis of variance. *$P < 0.05$ vs C, controls (vehicle). $^{\#}P < 0.05$ vs δ-TT-treated cells.

δ-TT triggers paraptosis in prostate cancer cells

Data reported above suggest that δ-TT may exert its activity by triggering non-canonical pro-death mechanisms in addition to apoptosis (see **Figure 2C**). Paraptosis represents an alternative cell death mechanism characterized by extensive vacuolization related to ER stress/mitochondria swelling. Paraptosis was also reported to be dependent on protein synthesis. We found that δ-TT (15 µg/mL, 18 hours) induces cytoplasmic vacuolization in both DU145 and PC3 cells (**Figure 7A**). By TEM analysis, we observed that untreated CRPC cells exhibit a normal appearance with normal mitochondria and ER with small profiles of cisternae. On the other hand, cells treated with δ-TT showed the presence of swollen damaged mitochondria with loss of/altered cristae, and ER cisternae dilation (**Figure 7B**, boxed areas). Moreover, pretreatment of CRPC cells with the ER stress inhibitor salubrinal (20 µM, 4 hours) markedly suppressed δ-TT-induced cytoplasmic vacuolization (**Figure 7C**), supporting the relationship between vacuoles and ER stress. To confirm the involvement of paraptosis in the anti-cancer activity of δ-TT, DU145 and PC3 cells were pretreated with cycloheximide (20 µM, 3 hours) and then with the tocotrienol. Translation inhibition strikingly suppressed the cytoplasmic vacuolization induced by δ-TT in both cell lines (**Figure 7C**). Finally, the effects of δ-TT (15 µg/ml, 24 hours) were analyzed on the expression of MAPK proteins, known to be involved in paraptosis. δ-TT (18 and 24 hours) increased the levels of both pJNK and pP38 kinases (**Figure 8**). These data support that paraptosis is involved in the anti-cancer activity of δ-TT in PCa cells.

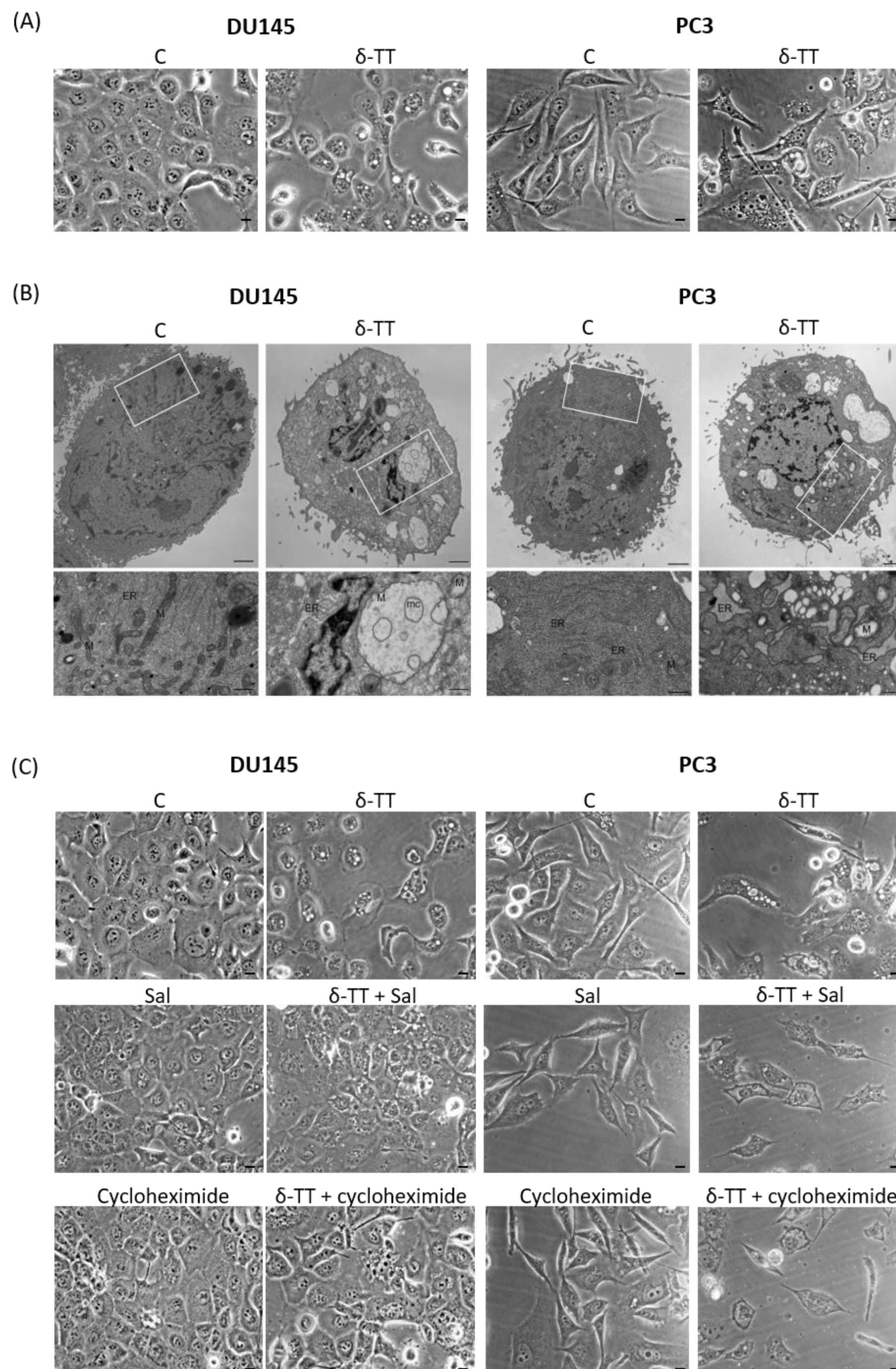

FIGURE 7. δ-TT triggers paraptosis in prostate cancer cells. In all these experiments, DU145 and PC3 cells were treated with δ-TT (15 µg/mL) for 18 h. **A**, light microscopy highlighting the presence of extensive cytoplasmic vacuolation in treated cells. Scale bars are 20 µm. **B**, TEM micrographs showing the presence of swollen damaged mitochondria (M), with loss or disintegrated cristae (mc) and endoplasmic reticulum (ER) cisternae dilatation (boxed areas), in both DU145 and PC3 treated cells. Scale bars are 2 µm, and 0.7 µm for photographs in boxed areas. **C**, light microscopy showing that pre-treatment of DU145 and PC3 cells with either the ER stress inhibitor salubrinal (Sal; 20 µM) for 4 h, or the translation inhibitor cycloheximide (20 µM) for 3 h, markedly suppresses cytoplasmic vacuolation in δ-TT-treated PCa cells. Scale bars are 20 µm.

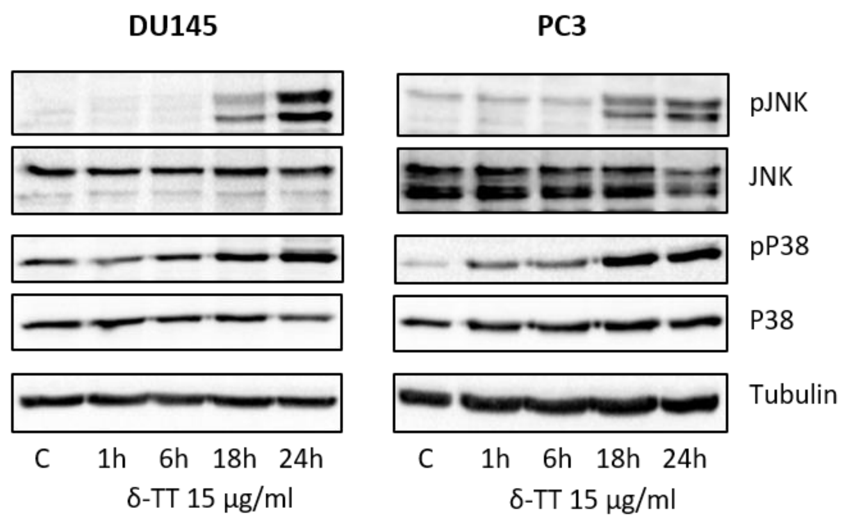

FIGURE 8. δ-TT increases the expression of proteins involved in the MAPK cascade in prostate cancer cells. DU145 and PC3 cells were treated with δ-TT (15 µg/mL) for 1-24 h. pJNK and pP38 expression levels were evaluated by Western blot analysis. Tubulin expression was assessed as a loading control. One representative of three different experiments performed is shown.

δ-TT inhibits mitochondrial respiration in prostate cancer cells

Since emerging evidence has highlighted the strict correlation between oxidative mitochondrial metabolism and cell death susceptibility [409,410], experiments were performed on PC3 and DU145 cells in order to clarify the role of mitochondria in the anti-tumor activity of δ-TT. Mitochondria are responsible for generating almost 90% of the energy needed to sustain cell proliferation. Thus, we first evaluated the effects of δ-TT on the mitochondrial respiration of CRPC cells, particularly on oxidative phosphorylation (OXPHOS) protein expression, O_2 consumption and ATP production. By Western blot analysis, we could observe that, in both PC3 and DU145 cells, the tocotrienol (15 μg/mL) significantly downregulates the expression of OXPHOS protein complexes, specifically complex I, III and IV (12-24 h, **Figure 9A**). δ-TT (15 μg/mL, 12 h) also significantly reduces O_2 uptake, in basal as well as in uncoupled (oligomycin, 10 μM) and maximal (carbonyl cyanide m-chlorophenyl hydrazine, CCCP, 10 μM) respiration conditions (**Figure 9B**). Notably, this resulted in mitochondrial membrane depolarization, as evidenced by cytofluorimetric analysis after staining with the fluorescent dye MitoTracker Orange CMTMRos (10 μM, 30 min, **Figure 9C**). Finally, we found that δ-TT (15 μg/mL), severely alters energy homeostasis, by causing massive ATP depletion (12 h, **Figure 10A**) and inducing a parallel activation (phosphorylation) of the energy sensor enzyme AMPK (6-24 h) (**Figure 10B**). Taken together, these data demonstrate that δ-TT exerts an energy-depleting effect on CRPC cells by impairing mitochondrial oxidative metabolism.

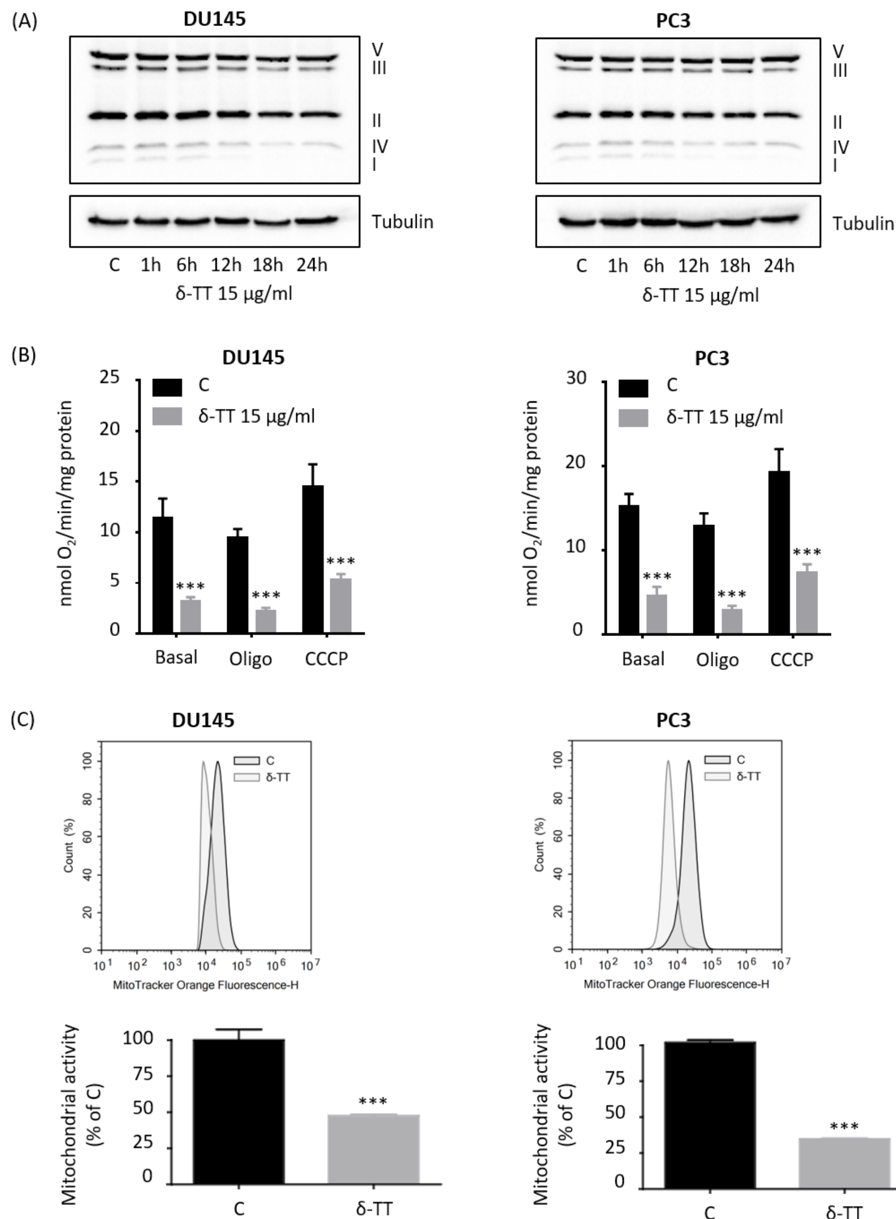

FIGURE 9. δ-TT targets mitochondrial oxidative metabolism in DU145 and PC3 prostate cancer cells. A, DU145 and PC3 cells were treated with δ-TT (15 µg/mL) for 1-24 h. Western blot analysis was performed to investigate the expression levels of OXPHOS proteins. Tubulin expression was evaluated as a loading control. One representative of three experiments performed is shown. **B**, DU145 and PC3 cells were treated with δ-TT (15 µg/mL) for 12 h; oxygen consumption rates were measured using a Clark electrode and they were evaluated in basal as well as in uncoupled (oligomycin, oligo) and maximal (carbonyl cyanide m-chlorophenyl hydrazine, CCCP) respiration conditions. One representative of three experiments performed is shown. Data represent mean values ± SEM and were analyzed by t-test. ***P < 0.001 vs C, controls (vehicle). **C**, DU145 and PC3 cells were treated with δ-TT (15 µg/mL) for 12 h; the mitochondrial activity was then evaluated by cytofluorimetric analysis after staining with the fluorescent dye MitoTracker Orange CMTMRos. One representative of three experiments performed is shown. Data represent mean values ± SEM and were analyzed by t-test. ***P < 0.001 vs C, controls (vehicle).

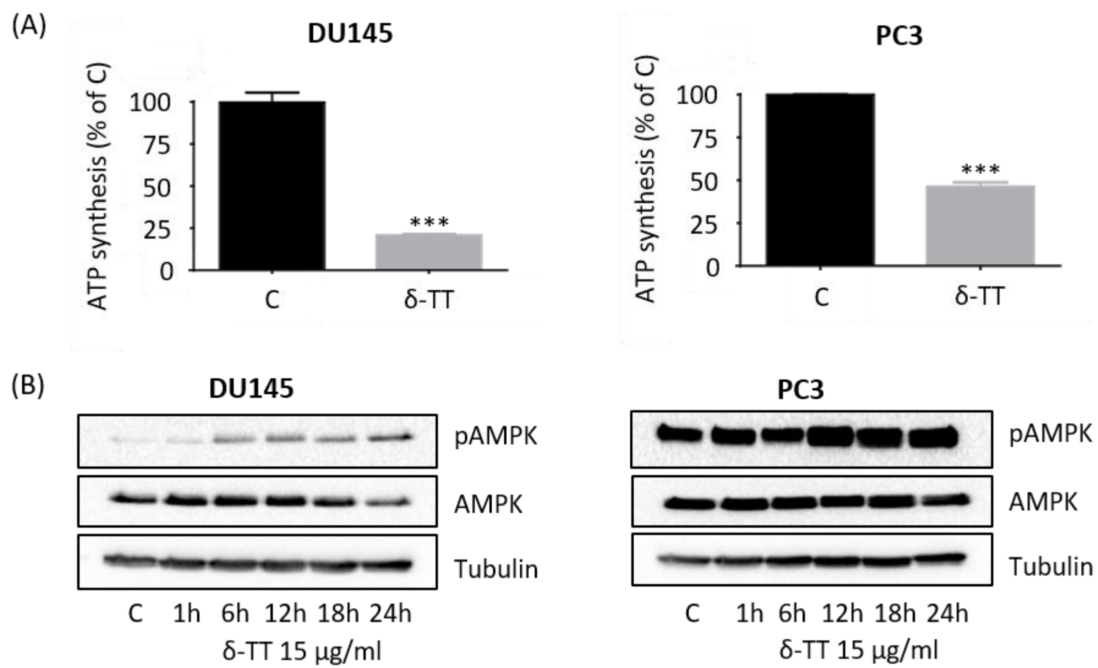

FIGURE 10. δ-TT exerts an energy-depleting effect on DU145 and PC3 prostate cancer cells. A, DU145 and PC3 cells were treated with δ-TT (15 μg/mL), and ATP levels were measured by colorimetric assay. One representative of three experiments performed is shown. Data represent mean values ± SEM and were analyzed by t-test. ***$P < 0.001$ vs C, controls (vehicle). **B**, Western blot analysis was performed to investigate the expression levels of pAMPK. Tubulin expression was evaluated as a loading control. One representative of three experiments performed is shown.

δ-TT impairs mitochondrial dynamics in prostate cancer cells

Mitochondrial morphology is tightly associated with mitochondrial health and function. Mitochondrial fusion correlates with efficient ATP production, whereas mitochondrial fission is related to reduced mitochondrial respiration [411,412]. As discussed above, the balance between these two opposite processes, and the consequent changes in mitochondrial morphology and localization, are finely regulated by two classes of proteins: fission (dynamin-related protein 1, DRP1) and fusion (optic atrophy 1, OPA1, mitofusin 1 and 2, MFN1 and MFN2) proteins [411,412].

By immunofluorescence studies, we found that δ-TT (15 µg/mL, 12 h) induces mitochondrial fission in both PC3 and DU145 cells, as evidenced by the intense punctuation of the MitoTracker probe (**Figure 11A**). In line with this observation, the tocotrienol (15 µg/mL, 24 h) also induced the cleavage of the long form of OPA1 in both cell lines, together with a decreased expression of MFN2 (**Figure 11B**, Western blot analysis) in PC3 cells. On the other hand, no significant change in Drp1 expression was observed (**Figure 11B**). Taken together, these results demonstrate that the mitochondrial fission-fusion machinery is directly targeted by δ-TT, confirming that an alteration of mitochondrial dynamics is associated with the δ-TT anti-tumor activity in CRPC cells.

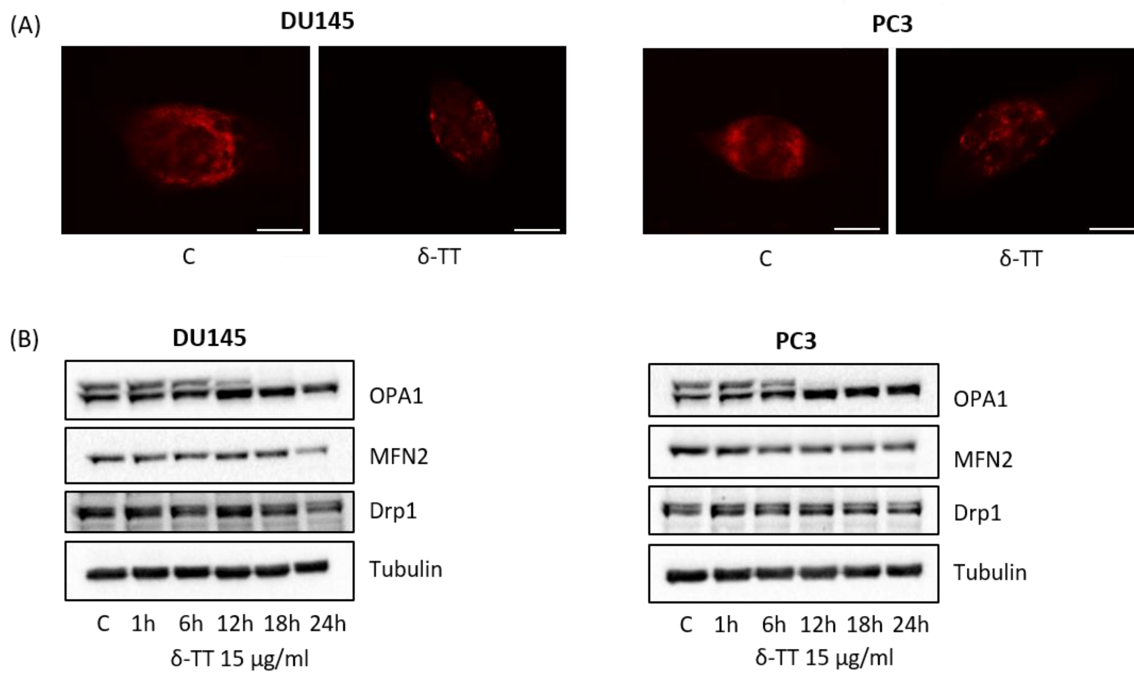

FIGURE 11. δ-TT impairs mitochondrial dynamics in DU145 and PC3 prostate cancer cells. A, DU145 and PC3 cells were treated with δ-TT (15 µg/mL), and the mitochondrial fragmentation was evaluated by immunofluorescence. One representative of three different experiments performed is shown. Scale bars are 20 µm. C, controls (vehicle). Scale bars are 20 µm. **B**, Western blot analysis was performed to investigate the expression levels of OPA1, MFN2 and Drp1. Tubulin expression was evaluated as a loading control. One representative of three experiments performed is shown.

Mitochondrial Ca^{2+} overload is involved in the pro-death activity of δ-TT in prostate cancer cells

As shown above, loss of mitochondrial potential and Ca^{2+} overload is associated with induction of apoptotic and paraptotic cell death. Various anti-cancer drugs, either synthetic or natural, were reported to activate the apoptotic and paraptotic pathways by inducing mitochondrial Ca^{2+} overload [223,273,274].

Here, we investigated the effects of δ-TT on cytoplasmic and mitochondrial Ca^{2+} levels (flow cytometry analysis), and the role of mitochondrial Ca^{2+} overload in the pro-death effects (apoptotic, paraptotic, autophagic) of this compound in PC3 and DU145 cells. We found that δ-TT (15 µg/mL, 12 h) significantly increases both cytoplasmic (Fluo-3 AM 5 µM, 30 min) and mitochondrial (Rhod-2 AM 5 µM, 30 min) Ca^{2+} levels (**Figure 12A**, **B**). Pretreatment of both cell lines with DIDS (disodium 4,4′-diisothiocyanostilbene-2,2′-disulfonate, 100 µM, 3h), the blocker of the VDAC channel, significantly counteracted the effects of the tocotrienol (15 µg/mL, 24 h) on: cell viability (**Figure 12C**), apoptosis (evaluated by analyzing the levels of cleaved caspase 3; **Figure 13A**), paraptosis (evaluated by analyzing intracellular vacuolation and JNK and p38 activation; **Figure 13B**, **C**), and autophagy (evaluated by analyzing the levels of LC3-II and p62; **Figure 13D**).

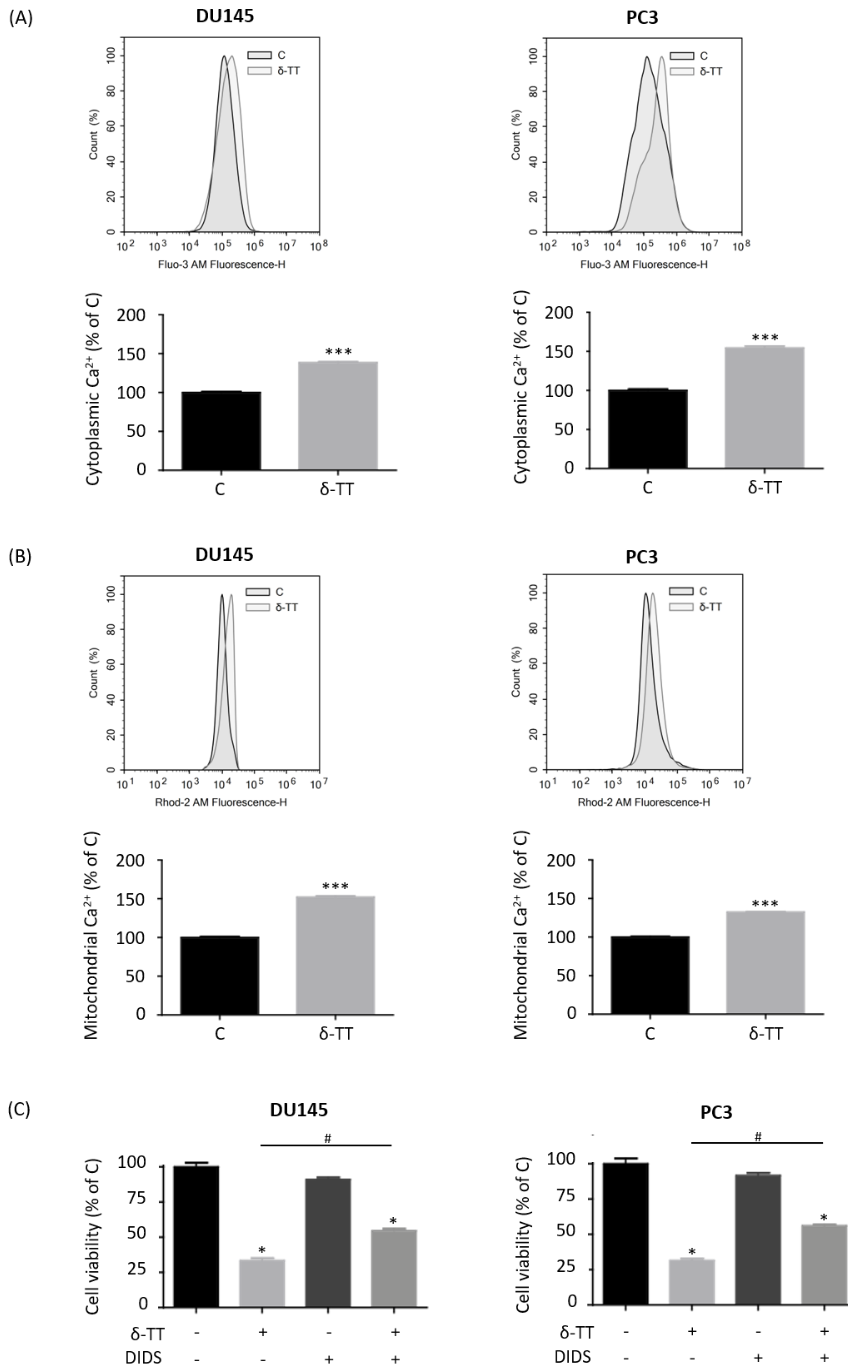

FIGURE 12. Mitochondrial Ca^{2+} overload is involved in the cytotoxicity of δ-TT in DU145 and PC3 prostate cancer cells. A and **B**, DU145 and PC3 cells were treated with δ-TT (15 µg/mL) for 12 h, and the cytoplasmic (Fluo-3 AM 5 µM, 30 min) and mitochondrial (Rhod-2 AM 5 µM, 30 min) Ca^{2+} levels were measured by cytofluorimetric analysis. Each experiment was repeated three times. Data represent mean values ± SEM and were analyzed by t-test. ***P < 0.001 vs C, controls (vehicle). **C**, Cells were pretreated with the VDAC inhibitor DIDS (100 µM) for 3 h, and then with δ-TT (15 µg/mL) for 24 h. Cell viability was assessed by MTT assay. Each experiment was repeated three times. Data represent mean values ± SEM and were analyzed by Bonferroni's test after one-way analysis of variance. *P < 0.05 vs controls (vehicle). #P < 0.05 vs δ-TT-treated cells.

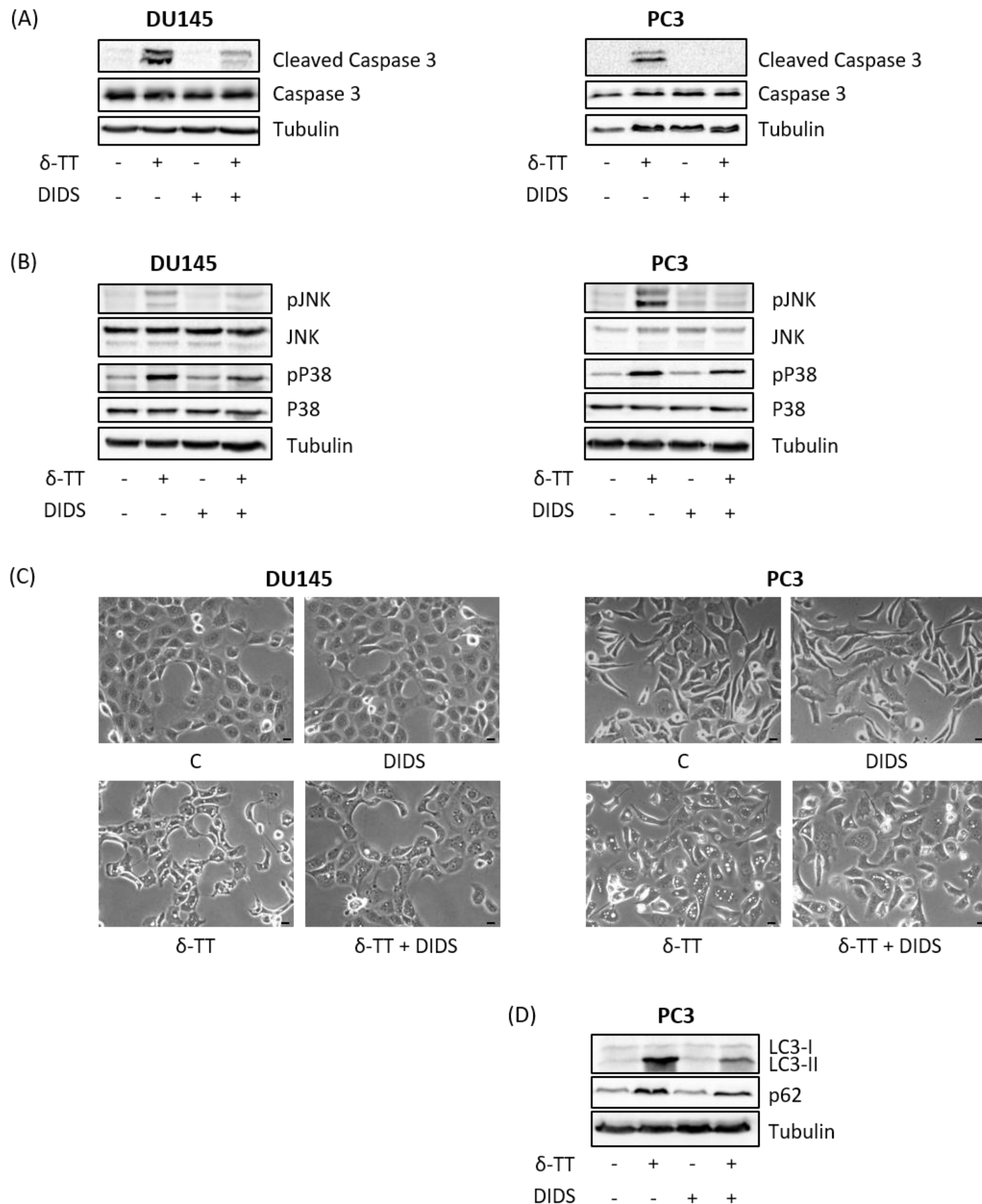

FIGURE 13. Mitochondrial Ca^{2+} overload is involved in the δ-TT-mediated apoptosis, paraptosis and autophagy in prostate cancer cells. A and **B**, Cells were treated with δ-TT (15 µg/mL) for 24 h in the presence or absence of DIDS (100 µM, 3 h). Caspase 3 cleavage and pJNK and pP38 levels were evaluated by Western blot analysis. Each experiment was repeated three times. **C**, Light microscopy showing that pretreatment of DU145 and PC3 cells with DIDS (100 µM, 3 h) markedly suppresses cytoplasmic vacuolation in δ-TT-treated PCa cells. Each experiment was repeated three times. Scale bars are 20 µm. **D**, PC3 cells were pretreated with DIDS (100 µM) for 3 h before tocotrienol treatment (15 µg/mL) for 24 h. The effects of the treatment were analyzed on the expression of autophagy markers by Western blot. One representative of three different experiments performed is shown.

The ROS/Akt pathway is involved in the pro-death activity of δ-TT in PC3 prostate cancer cells

It is well accepted that high intracellular levels of ROS can induce oxidation of macromolecules (nucleic acids, proteins and lipids) and damage cell membranes and organelles, triggering cell death pathways such as apoptosis and paraptosis. The deep interplay between mitochondrial dysfunction and ROS generation has been discussed in detail. Moreover, it is known that cancer cells possessing high endogenous ROS levels are more susceptible to ROS-inducing treatments; indeed, different anti-cancer drugs trigger apoptosis and paraptosis in cancer cells by inducing ROS generation [280-288].

Here, we analyzed the effects of δ-TT on intracellular ROS production (flow cytometry analysis after staining with the fluorescent dye DCFDA 10 μM for 30 min) and the role of ROS formation in the anti-cancer activity of this compound in CRPC cells. We observed that δ-TT (15 μg/mL, 12 h) significantly increased ROS levels in both PC3 and DU145 cells, although this effect was more evident in PC3 cells (**Figure 14A**). Indeed, we then demonstrated that pretreatment of cancer cells with NAC (N-acetyl-L-cysteine, 4 mM**,** 2 h), the known ROS scavenger, significantly counteracted the inhibitory activity of δ-TT (15 μg/mL, 24 h) on cell viability in PC3 but not in DU145 cells (**Figure 14B**). These data confirm previous observations showing that ROS-mediated cell death can be induced in PC3 but not in DU145 cells [413,414].

Based on these observations, subsequent experiments were carried out in PC3 cells. We demonstrated that, in these cells, pretreatment with NAC significantly inhibits the effects of δ-TT (15 μg/mL, 24 h) on apoptosis (**Figure 15A**), paraptosis (**Figure 15B**, **C**), and autophagy (**Figure 15D**).

It is known that the pro-tumor activity of high ROS levels is associated with various signaling pathways, including the PI3K/Akt signaling pathway. Thus, we investigated the effects of δ-TT (15 μg/mL, 24 h) on Akt/mTOR activation in CRPC cells and the involvement of ROS in these effects. First, we found that pAkt (the activated phosphorylated form) is constitutively expressed in PC3, but not in DU145 PCa cells (**Figure 16A**). In addition, in PC3 cells, δ-TT significantly reduced pAkt levels in a time-dependent manner (**Figure 16B**), and this effect was completely counteracted by

pretreatment of the cells with the ROS scavenger NAC (4 mM, 2 h) (**Figure 16C**). These data confirm that constitutive activation of Akt in cancer cells confers them a high vulnerability to anti-cancer compounds specifically targeting ROS formation.

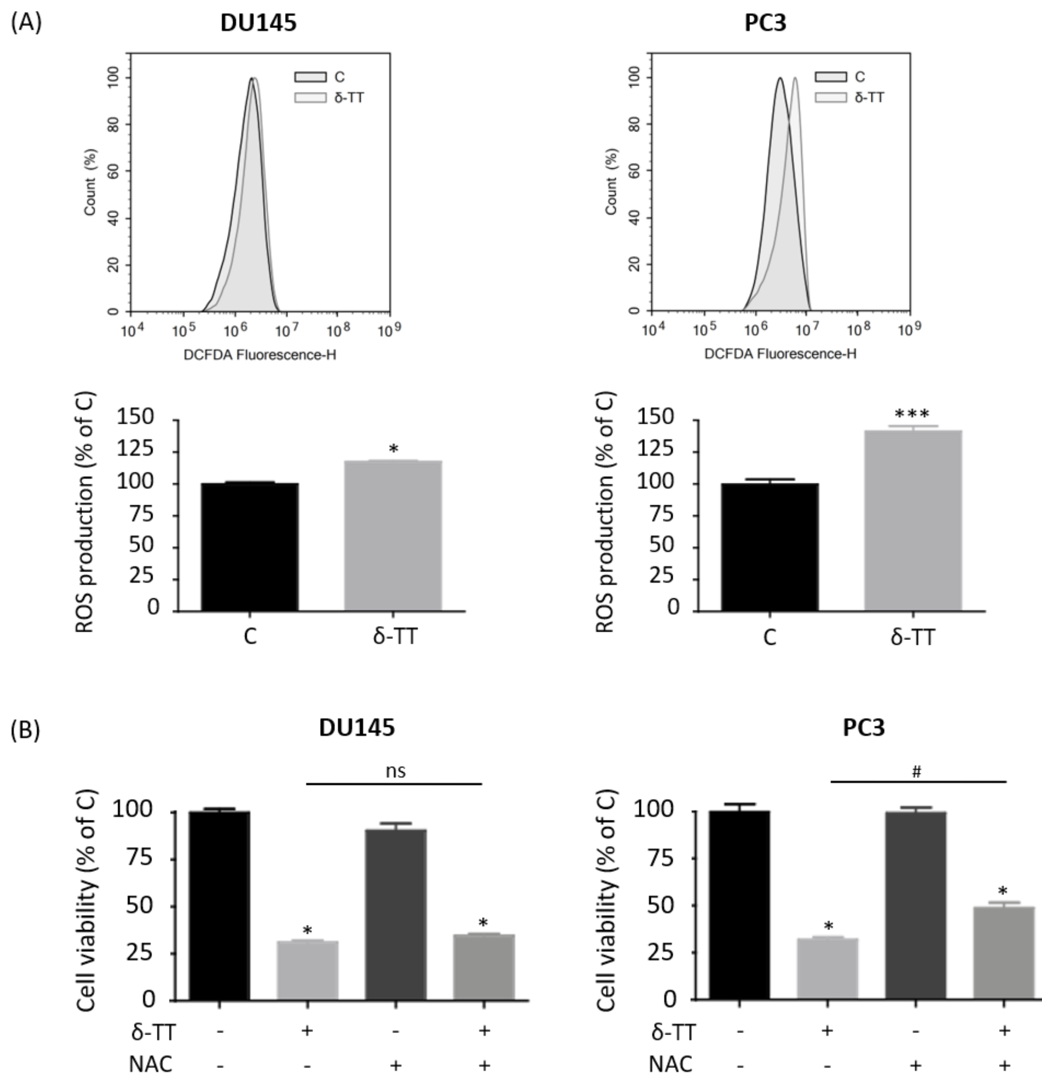

FIGURE 14. Oxidative stress is involved in the cytotoxicity of δ-TT in DU145 and PC3 prostate cancer cells. A, DU145 and PC3 cells were treated with δ-TT (15 µg/mL) for 12 h, and the ROS production was measured by cytofluorimetric analysis (DCFDA, 10 µM, 30 min). Each experiment was repeated three times. Data represent mean values ± SEM and were analyzed by t-test. ***P < 0.001 vs C, controls (vehicle). **B**, Cells were pretreated with the ROS scavenger NAC (N-acetyl-L-cysteine, 4 mM**)** for 2 h, and then with δ-TT (15 µg/mL) for 24 h. Cell viability was assessed by MTT assay. Each experiment was repeated three times. Data represent mean values ± SEM and were analyzed by Bonferroni's test after one-way analysis of variance. *P < 0.05 vs controls (vehicle). #P < 0.05 vs δ-TT-treated cells.

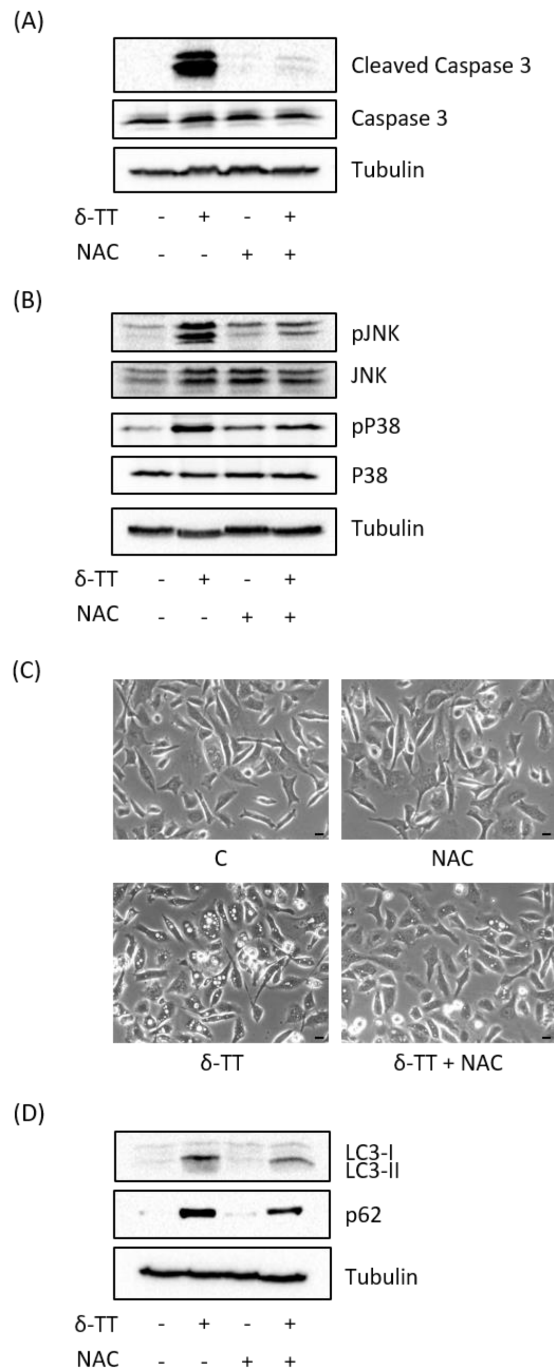

FIGURE 15. ROS generation is involved in the δ-TT-mediated apoptosis, paraptosis and autophagy in prostate cancer cells. A and **B**, Cells were treated with δ-TT (15 µg/mL) for 24 h in the presence or absence of NAC (4 mM, 2 h). Caspase 3 cleavage and pJNK and pP38 levels were evaluated by Western blot analysis. Each experiment was repeated three times. **C**, Light microscopy showing that pretreatment of DU145 and PC3 cells with NAC (4 mM, 2 h) markedly suppresses cytoplasmic vacuolation in δ-TT-treated PCa cells. Each experiment was repeated three times. Scale bars are 20 µm. **D**, PC3 cells were pretreated with NAC (4 mM) for 2 h before tocotrienol treatment (15 µg/mL) for 24 h. The effects of the treatment were analyzed on the expression of autophagy markers by Western blot. One representative of three different experiments performed is shown.

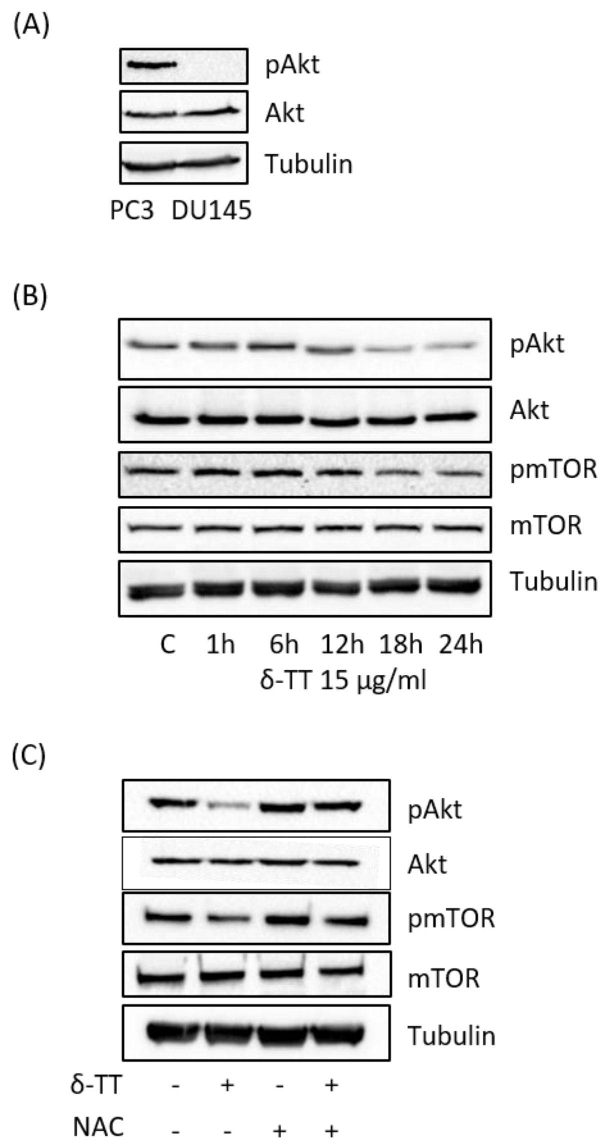

FIGURE 16. The ROS/Akt pathway is involved in the pro-death activity of δ-TT in PC3 prostate cancer cells. A, pAkt expression was evaluated in PC3 and DU145 cells by Western blot analysis. Each experiment was repeated three times. **B**, PC3 cells were treated with δ-TT (15 μg/mL) for 24 h. pAkt and pmTOR levels were evaluated by Western blot analysis. Each experiment was repeated three times. **C**, PC3 cells were pretreated with NAC (4 mM) for 2 h before tocotrienol treatment (15 μg/mL) for 24 h. The effects of the treatment were analyzed on the expression of pAkt and pmTOR by Western blot. One representative of three different experiments performed is shown.

δ-TT induces Ca^{2+}- and ROS-mediated mitophagy in PC3 prostate cancer cells

Increasing evidence suggests that dysfunctional/damaged mitochondria are eradicated through a specific degradation pathway, known as 'mitophagy'.

Based on these data, we investigated whether δ-TT might induce mitophagy in PCa cells. These experiments were performed in PC3 cells, since DU145 cells are autophagy-defective. By immunofluorescence studies, in δ-TT-treated (15 µg/mL, 12 h) PC3 cells, we demonstrated the presence of autophagic vesicles containing mitochondria (**Figure 17A**). In line with this observation, by TEM analysis we showed the presence of mitochondria-like structures inside autophagic vesicles (**Figure 17B**). To further confirm the induction of mitophagy by δ-TT, we could demonstrate that the tocotrienol (15 µg/mL, 24 h) time-dependently increase the expression of the PINK1 kinase, while Parkin levels were not affected by the treatment, as expected (**Figure 17C**).

Given the role of Ca^{2+} and ROS in the pro-death activity of δ-TT in PCa cells, we finally investigated whether δ-TT-induced mitophagy might also be mediated by Ca^{2+} and ROS levels. PC3 cells were pretreated with either the blocker of VDAC channel DIDS (100 µM, 3 h) or the ROS scavenger NAC (4 mM, 2 h) before δ-TT treatment (15 µg/mL, 24 h). As shown in **Figure 17D** and **E**, both DIDS and NAC significantly counteracted the δ-TT-induced expression levels of PINK1.

Taken together, these results demonstrate that δ-TT triggers Ca^{2+}- and ROS-mediated autophagic clearance of mitochondria in PC3 cells.

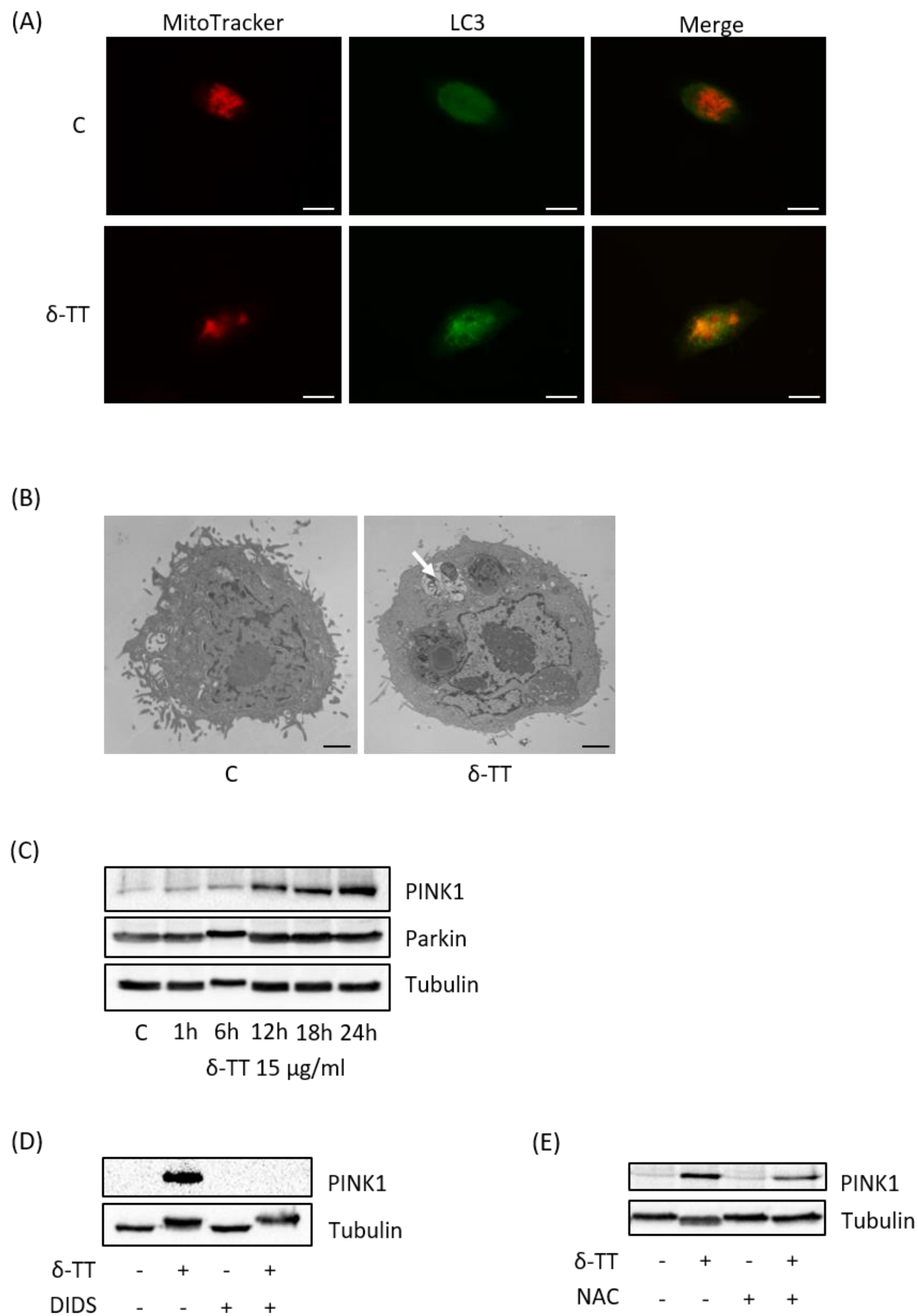

FIGURE 17. δ-TT induces Ca^{2+}- and ROS-mediated mitophagy in PC3 prostate cancer cells. A, PC3 cells were treated with δ-TT (15 µg/mL) for 12 h; the induction of mitophagy was then evaluated by immunofluorescence analysis. One representative of three experiments performed is shown. Scale bars are 20 µm. **B**, TEM micrograph showing the presence of autophagolysosomes containing damaged mitochondria in PC3 treated cells. Scale bars are 2 µm. **C**, PC3 cells were treated with δ-TT (15 µg/mL) for 24 h. PINK1 and Parkin levels were evaluated by Western blot analysis. Each experiment was repeated three times. **D** and **E**, PC3 cells were pretreated with NAC (4 mM) for 2 h before tocotrienol treatment (15 µg/mL) for 24 h. The effects of the treatment were analyzed on the expression of PINK1 by Western blot. One representative of three different experiments performed is shown.

DISCUSSION

It is accepted that TTs are associated with significant anti-cancer properties [415,416]. Although most of the studies so far reported were performed with γ-TT, δ-TT was shown to be the most effective vitamin E isoform in triggering cell death in PCa cells [316,417]; however, the molecular mechanisms/targets of this activity are poorly clarified.

Here, we dissected the molecular mechanisms underlying the anti-tumor/pro-apoptotic activity of δ-TT in CRPC cells (DU145 and PC3); the possible involvement of paraptosis in its activity was also investigated.

We confirmed that δ-TT exerts a significant anti-tumor/cytotoxic activity on CRPC cells, by decreasing cell viability, increasing the dead/live cell ratio and reducing the viability of colony-forming cells. Interestingly, the tocotrienol reduced the viability of RWPE-1 cells only slightly and at the highest dose. We also demonstrated that δ-TT triggers apoptosis by increasing the levels of cleaved caspase 3 and PARP and inducing the release cytochrome *c* from mitochondria into the cytoplasm. We finally confirmed the involvement of the intrinsic apoptosis in the activity of δ-TT by showing that pretreatment of the cells with the pan-caspase inhibitor Z-VAD-FMK significantly counteracts its cytotoxic effects.

These data agree with previous observations showing that δ-TT induces cell death in PCa cells [316,417] and suppresses the survival of the stem-like cells subpopulation of PC3 cells [321]; similar results were reported for γ-TT [315,415,418].

In line with these data, the anti-cancer activity of TTs (specifically γ- and δ-TT) was reported in a wide range of tumors [415,419,420]. To get further insights into the mechanisms and targets of the δ-TT anti-tumor activity in CRPC cells, we concentrated our studies on the ER stress and autophagy pathways. We observed that, in both DU145 and PC3 cells, δ-TT induces the expression of BiP, peIF2α and IRE1α. δ-TT also induced the expression/activation of the transcription factors ATF4 and CHOP (pointing out their cytoplasmic-to-nuclear localization). It is known that the peIF2α/ATF4 pathway activates CHOP, a transcription factor that is also activated by IRE1α [421,422]. These results demonstrate that, in CRPC cells, δ-TT triggers the main ER stress branches, leading to the activation of CHOP, deeply involved in the ER stress-related apoptosis. To confirm

the involvement of the ER stress pathway in the activity of δ-TT, we pretreated the cells with two ER stress inhibitors, salubrinal and 4-PBA. Both inhibitors significantly reverted the pro-apoptotic effect of δ-TT, as assessed in terms of cleavage of caspase 3 and PARP as well as of cell viability, indicating that ER stress mediates its anti-cancer activity.

We also investigated whether autophagy might be induced by δ-TT in CRPC cells. First, we demonstrated that the tocotrienol markedly increases the expression of autophagy-related proteins, such as LC3 (increased LC3-II/LC3-I ratio) and SQSTM1/p62 and their accumulation into autophagosomes in PC3, but not in DU145 cells (previously reported to be autophagy-defective) [408]. Thus, the involvement of autophagy in the anti-tumor activity of δ-TT was further investigated in PC3 cells. By TEM, we demonstrated the presence of autophagosomes and autophagolysosomes in δ-TT-treated cells. Pretreatment of the cells with an early stage autophagy inhibitor (3-methyladenine, 3-MA) significantly counteracted, while their pretreatment with late stage autophagy inhibitors (chloroquine, CQ, and bafilomycin, Baf) markedly increased δ-TT -induced LC3-II and SQSTM1/p62 expression.

These data support that δ-TT triggers an autophagic flux in PC3 cancer cells. We further showed that in PC3 cells the autophagic pathway is linked to ER stress, since pretreatment of the cells with the ER stress inhibitors markedly prevented δ-TT-induced LC3-II and SQSTM1/p62 overexpression. Finally, we demonstrated that the autophagy inhibitor 3-MA significantly counteracts the effects of δ-TT on apoptosis markers as well as on cell viability.

These data demonstrate that, in CRPC cells possessing an efficient autophagic pathway, δ-TT induces apoptosis by triggering the ER stress-related pro-death autophagic flux. On the other hand, only the ER stress pathway is involved in the activity of δ-TT in autophagy-defective cells.

To our knowledge, this is the first report describing the involvement of the ER stress-autophagy in the anti-cancer activity of δ-TT in PCa cells. γ-TT was shown to concurrently trigger ER stress and autophagy in inducing apoptosis in breast cancer cells [338,339]. TTs (specifically γ- and δ-TT) were shown to induce apoptosis by triggering the ER stress branches in cervical cancer [360], breast cancer [337] and melanoma cells [392]. In line

with these observations, both the ER stress and the autophagy pathways were reported to mediate the anti-cancer activity of several natural compounds [423,424].

Here, we also observed that abrogation of apoptosis by the pan-caspase inhibitor Z-VAD-FMK significantly, but not completely, reverted the cytotoxic effect of δ-TT on CRPC cells. Thus, an additional programmed cell death modality might be involved in the activity of the tocotrienol. Paraptosis, necroptosis, mitotic catastrophe, anoikis were reported to be typical of apoptosis-resistant tumor cells and to mediate the cytotoxic effects of anti-cancer compounds [219,425,203]. This makes these types of cell death a promising target for novel therapeutic strategies [203]. Among them, paraptosis is characterized by: intense cytoplasmic vacuolation, correlated with ER stress and mitochondrial swelling/dilatation; *de novo* protein synthesis; involvement of JNK and p38 kinases [219,201,203]. We observed that δ-TT induces morphological changes, with an intense cytoplasmic vacuolation in both CRPC cells. In δ-TT-treated cells, we pointed out: by TEM, a significant swelling of mitochondria and dilatation of the ER cisternae; by light microscopy, a cytoplasmic vacuolation that was markedly inhibited in the presence of salubrinal or cycloheximide; by Western blot, increased expression of the active forms of JNK and p38. These data support that, in addition to apoptosis, the non-canonical cell death paraptosis is involved in the anti-tumor activity of δ-TT in CRPC cells.

To further clarify the molecular mechanisms underlying both the pro-apoptotic and pro-paraptotic effects of δ-TT, we focused our attention on the study of mitochondrial metabolism. Indeed, in the last years mitochondria have emerged as important pharmacological targets because of their key role in cellular proliferation and death [409,410]. Here, we observed that, in both DU145 and PC3 cells, the tocotrienol treatment resulted in a rapid loss of mitochondrial membrane potential, accompanied by downregulation of OXPHOS protein expression levels and reduction of oxygen consumption. As direct consequence of mitochondrial damage, a significant decline in cellular ATP levels and a parallel activation of the energy sensor AMPK were also found. Taken together, these data demonstrate that δ-TT severely alters mitochondrial homeostasis in CRPC cells, also exerting an energy-depleting effect on them.

We also evaluated the effects of δ-TT on mitochondrial dynamics. In particular, we demonstrated that the tocotrienol directly target the fission-fusion machinery in CRPC cells, inducing mitochondrial fragmentation associated with OPA-1 cleavage and MFN2 downregulation.

Since mitochondrial dysfunction is often associated with Ca^{2+} overload and oxidative stress, we measured Ca^{2+} and ROS levels in CRPC cells treated with δ-TT. A significant increase of both cytoplasmic and mitochondrial Ca^{2+}, as well as of ROS levels, was observed in DU145 and PC3 cells after δ-TT treatment. Using the VDAC blocker4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) and the ROS scavenger N-acetyl-l-cysteine (NAC), we investigated the role of Ca^{2+} and ROS in the induction of cell death: interestingly, DIDS pretreatment significantly rescued cell viability of both DU145 and PC3 cells, while NAC pretreatment was effective only on the more oxidatively stressed PC3 cells. In particular, Ca^{2+} overload was shown to be directly involved in apoptosis, paraptosis and autophagy activation in CRPC cells; similarly, ROS generation was responsible for apoptotic, paraptotic and autophagic PC3 cell death.

In order to elucidate the molecular mechanisms of the ROS-mediated cell death induced by δ-TT in PC3 cells, we focused our studies on the Akt signaling pathway. Indeed, it is known that this cascade is mainly responsible for ROS generation in overproliferative cancer cells, making them more susceptible to oxidative stress-related damage [285-288]. Here, we first confirmed that Akt protein is constitutively activated in PC3 cells but not in DU145 cells. Then, we observed that δ-TT treatment lead to a time-dependent downregulation of this pathway in PC3 cells. In addition, NAC pretreatment significantly reverted the tocotrienol-induced reduction in Akt levels. Taken together, these results highlight that δ-TT can inhibit the Akt cascade through ROS generation in PC3 cells.

Dysfunctional mitochondria are usually eliminated via mitophagy [275]. In PC3 cells, we found that δ-TT can also activate the PINK1-mediated mitophagic flux.

In conclusion, these data demonstrate that, in CRPC cells, δ-TT exerts an anti-cancer activity by triggering both apoptosis, involving the ER stress/autophagy axis, and paraptosis. In particular, we showed that it can selectively alter mitochondrial

morphology and function and induce Ca^{2+} overload- and oxidative stress-mediated CRPC cell death, providing novel mechanistic insights into its anti-tumor activity.

Despite all these encouraging observations, the clinical data so far available are still incomplete and several important questions remain to be addressed about the role of TTs in cancer patients, especially as regards their pharmacokinetics and bioavailability. Thus, clinical trials aimed to clarify the TT anti-tumor effectiveness are urgently needed.

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