Università degli Studi di Palermo

AREA QUALITÀ, PROGRAMMAZIONE E SUPPORTO STRATEGICO
SETTORE STRATEGIA PER LA RICERCA
U. O. DOTTORATI

D067 – Technologies and Sciences for Human Health
Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche (STEBICEF)
SSD: MED/13

RESISTANCE TO VANDETANIB IN MEDULLARY THYROID CARCINOMA: POSSIBLE NEW TREATMENT

LA DOTTORANDA
Concetta Baiamonte

IL COORDINATORE
Prof. Vincenzo Cavalieri

IL TUTOR
Prof.ssa Carla Giordano

CICLO XXV
ANNO 2023

INDEX

SUMMARY

Thyroid Cancer (TC) accounts for about 3% of all cancers and is the most common malignant endocrinological tumor. One of the most malignant thyroid cancers is Medullary Thyroid Carcinoma (MTC), the only parafollicular C-cell-derived tumor. MTC accounts for about 5% of all thyroid cancers and is sporadic in about 75% of cases. In the last few decades, several genetic alterations have been identified in different molecular pathways and associated with tumor aggressiveness and progression (BRAF, RAS, VEGFR and RET). RET is a proto-oncogene (10q11.2), which codes for a transmembrane tyrosine kinase receptor and is expressed on tissues deriving from the neural crest including thyroid C cells but not in normal thyroid follicular cells. Activation mutations involving RET have been identified in about 98% of hereditary MTC and about 50% of sporadic MTC. In all cases, the oncogenic protein RET hyper-activates a complex network pathway (i.e. RAS/ERK pathway and PI3K/AKT pathway). Preclinical and clinical studies have demonstrated that therapy based on RET inhibition with MultiKinase Inhibitors (MKIs) and/or with RET-specific inhibitors, a TyrosineKinase Inhibitors (TKI), may be a promising strategy for the treatment of cancers in which this oncogene is involved. In 2011 Vandetanib (Caprelsa, ZD6474; AstraZeneca) was approved by the U.S. Food and Drug Administration, as RET kinase inhibitor for the treatment of inoperable progressive MTC or metastatic patients. Clinical experience with RET TKIs is limited, however, lessons learned from other tumor types raised the hypothesis that cancer patients commonly could develop TKI resistance. Polyphenols, represent the main target of cancer research as they show potential for becoming superior agents for preventing and treating various malignancies. A combination of cytoprotective effects toward normal cells and cytotoxic effects toward cancerous cells thus represents the main advantage of polyphenols as anticarcinogenic agents. In recent years, many different combined compounds and therapeutic approaches have been used to treat devastating diseases such as cancer. The main aim is to achieve a synergistic therapeutic effect, to reduce the dose and toxicity, and to minimize or delay the induction of drug resistance such that the combinations would add benefits. In *in vitro* studies, the Combination Index (CI) method is a quantitative representation of the pharmacologic interaction between two drugs. Several important and clinically relevant interactions between polyphenols and conventional drugs have been reported over the last few years. Moreover, many *in vitro* and *in vivo* studies suggest that polyphenols can be used as

chemosensitizers when combined with chemotherapeutic agents (i.e. Doxorubicin, Cisplatin and Taxol). There are many molecular targets on which polyphenols act as chemosensitizing agents, they are involved in cell cycle regulation, apoptosis, oxidation and angiogenesis. They work by up-regulating factors such as Caspase-3, the p21 protein and/or PTEN (Phosphatase and TENsin homolog), and down-regulating factors such as NF-kB (Nuclear Factor kappa B), Survivin, metalloproteases and CREB (cAMP Response Element Binding protein).

Thus, overcoming chemoresistance to Vandetanib *in vitro* through also the use of polyphenols appears of great clinical relevance.

In this study, it is proposed to evaluate the possible secondary resistance mechanisms to Vandetanib and chemosensitizing to Vandetanib by polyphenols human MTC cell line chemoresistant.

We proceeded to pursue these goals to generate *in vitro* and characterize MTC cells resistant to Vandetanib analyzing some of the possible molecular mechanisms involved in this resistance; To evaluate the stemness component in the MTC Vandetanib-resistant cells during the process of resistance acquisition; To investigate whether molecules belonging to the polyphenol class could sensitize Vandetanib resistant MTC cell line.

It was demonstrated that the constant exposure to increasing concentrations of Vandetanib results in Vandetanib-resistant cells partially sensitive to chemosensitization with the polyphenols.

BACKGROUND

Thyroid gland

The thyroid gland is a vital hormone gland due to the major role that it has in the metabolism, growth and development of the human body. By releasing a steady amount of thyroid hormones in the bloodstream it helps with the regulation of many body functions. Glandula thyreoidea is found at the front of the neck, under the voice box. It is butterfly-shaped: the two side lobes lie against and around the windpipe and are connected at the front by a narrow strip of tissue (1). The weight of the thyroid is between 20 and 60 grams on average and is surrounded by two fibrous capsules.

The thyroid tissue itself consists of a lot of small individual lobules, enclosed in thin layers of connective tissue. These lobules contain a great number of small vesicles. These vesicles are called follicles and store thyroid hormones, in the form of little droplets (2).

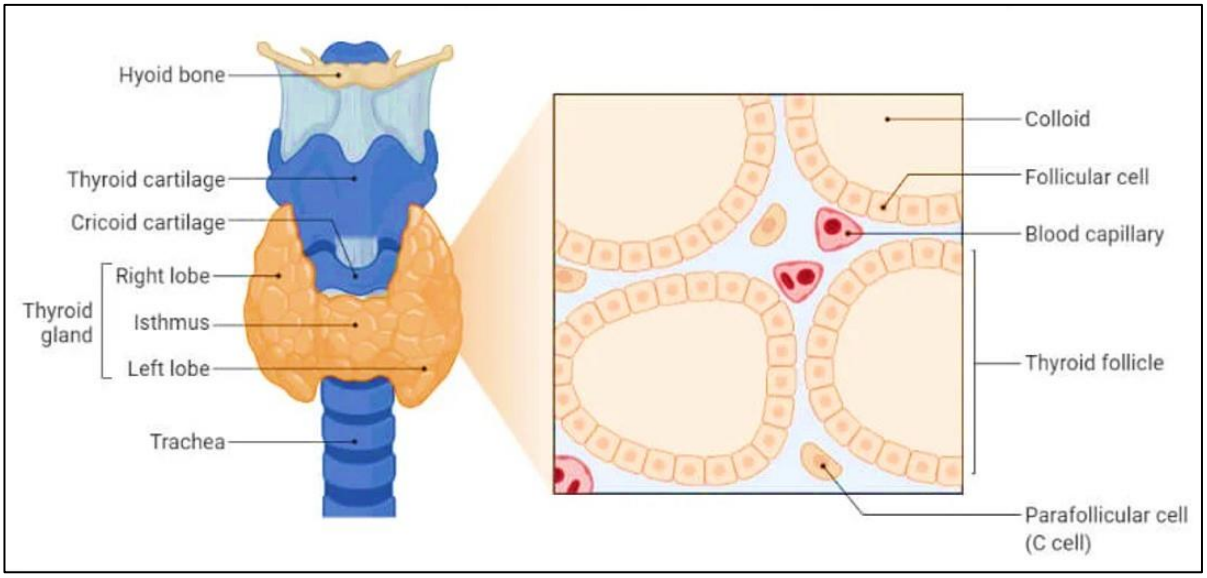


Fig.1: The Thyroid Gland: Location, Anatomy, Organization and Hormones. Adopted by: Anupama Sapkota. Thyroid Gland- Definition, Structure, Hormones, Functions, Disorders, The Biology Notes. https://thebiologynotes.com/thyroid-gland/ (3).

Follicular Cells produces Thyroxine (referred to as T4), and Triiodothyronine (referred to as T3). The thyroid gland releases in the bloodstream T4 which constitutes about 80% of the secreted thyroid hormones and T3 which constitutes about 20%. Once secreted, the T4 is converted into the T3 (which accounts for most of the T3 in the body) by specific enzymes in other tissues like the liver or

kidneys. In addition, there are other hormone-producing cells within the thyroid gland a Parafollicular Cell called C-cells. These cells produce Calcitonin which plays an important role in regulating calcium and phosphate levels in the blood (4).

Thyroid Cancer

Thyroid cancer (TC) represents about 3% of all cancers. It is the most common malignant endocrinological tumor (5) with 586.000 new patients estimated in 2020 (6). Thyroid cancer originates from follicular epithelial cells or parafollicular C cells. Thyroid cancer originates from follicular epithelial cells are classified into four histological types: papillary thyroid cancer (PTC 80-85%), follicular thyroid cancer (FTC 10-15%), poorly-differentiated thyroid cancer (PDTC, <2%), and anaplastic thyroid cancer (ATC, <2%). PTC and FTC are well-differentiated thyroid cancers (WDTC) accounting for the majority of thyroid cancer cases (7). Medullary thyroid cancer (MTC), arising from the parafollicular cells (C cells), represents 4% of all thyroid cancers. MTC can be both hereditary (25%) or sporadic (75%) (8). Hereditary MTC including familial MTC (FMTC) and multiple endocrine neoplasia type 2 (MEN). FMTC is characterized by MTC alone, recur in several relatives of the same family. In MEN-2A, thyroid carcinoma can be associated with additional endocrine neoplasia such as pheochromocytoma and/or hyperparathyroidism; in MEN-2B, thyroid carcinoma can be associated with non-endocrine diseases such as mucosal neuromas, megacolon, marfanoid habitus and skeletal abnormalities (9).

In the last 30 years, the availability of the genome sequence has greatly helped the progress in elucidating the molecular mechanisms underlying TC.

Driver mutations, identified in more than 90% of TC, provide a selective growth advantage thus promoting cancer development. The molecular pathogenesis of TC involves dysregulation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K)/AKT pathways. For PTC initiation, the MAPK activation is crucial, through point mutations of the BRAF and RAS genes or gene fusions of RET/PTC and Tropomyosin Receptor Kinase (TRK). On the other hand, PI3K/AKT activation is believed to be critical in FTC initiation. This activation can be triggered by activating mutations in RAS, PIK3CA, and AKT1 as well as by the inactivation of PTEN, which negatively regulates this pathway. TC progression and dedifferentiation to PDTC and ATC involve several

additional mutations affecting other cell signaling pathways, such as p53 and Wnt/β-catenin. Recently, TERT promoter mutations have been described in all the histological TC types, having a significantly higher prevalence in aggressive and undifferentiated tumors. This indicates their role in TC progression (10) Mutations in RET (REarranged during Transfection) proto-oncogene account for most MTC cases. They can occur either sporadically or as inherited germline events in the MEN-2A and MEN-2B syndromes. Moreover, a minority of sporadic MTC are caused by H-, K-, and N-RAS mutations (11).

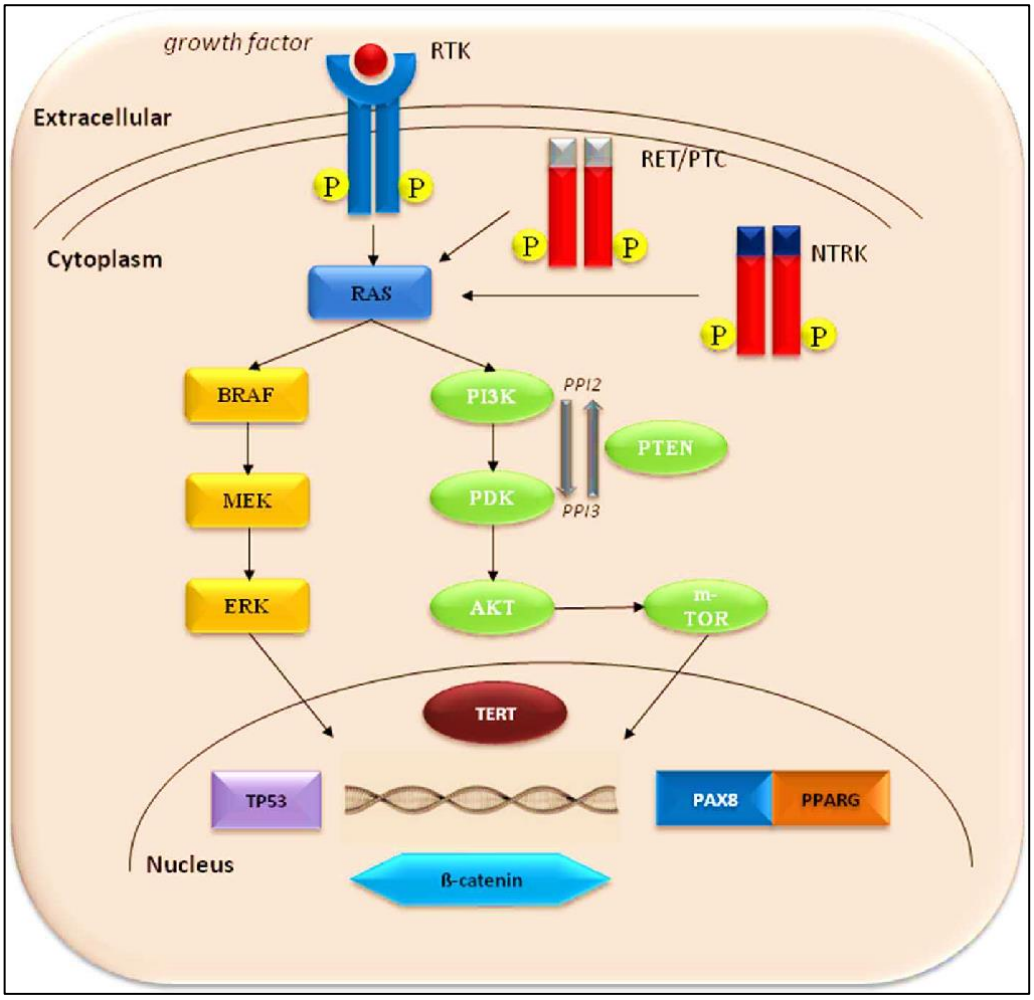


Fig.2: The molecular pathogenesis of thyroid cancer: Dysregulation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K)/AKT pathways. Adopted by: Prete A, Borges de Souza P, Censi S, Muzza M, Nucci N, Sponziello M. Update on Fundamental Mechanisms of Thyroid Cancer. Front Endocrinol (Lausanne). 2020 Mar 13;11:102. doi: 10.3389/fendo.2020.00102. PMID: 32231639; PMCID: PMC7082927 (10).

RET (REarranged during Transfection)

RET is a proto-oncogene (10q11.2) which codes for a transmembrane tyrosine kinase receptor and is expressed on tissues deriving from the neural crest including thyroid C cells but not in normal thyroid follicular cells (12). RET gene which is subjected to alternative splicing that gives origin to three functional isoforms: RET51, RET9, and RET43. RET9 is expressed in several human tissues while RET51 is only expressed in some of them. Moreover, when compared, the RET51 expression has been found to be lower than the RET9 expression. Conversely, RET51 isoform expression has been reported to be higher in MTC than in PTC, in more aggressive forms of pancreatic cancer, and pheochromocytoma. This suggests that this isoform has a specific role in determining the aggressiveness of a tumor. As a matter of fact, due to the differentiation in the biochemical and biological properties of these two isoforms, they play distinct roles in tumorigenesis and/or development (13).

The RET protein is comprised of an extracellular domain, a transmembrane domain, and an intracellular tyrosine kinase domain. The extracellular domain contains four cadherin-like domains (CLD1-4), a calcium-binding site, and a cysteine-rich domain. The intracellular region contains a tyrosine kinase domain and tyrosine phosphorylation sites located next to the C terminal region, where two major isoforms, RET9 and RET51, are positioned due to alternative splicing. There are four known RET ligands, artemin (ARTN), glial cell line-derived neurotrophic factor (GDNF) neurturin (NRTN), and persephin (PSPN), and four co-receptors that form GDNF family receptor-α 1-4 (GFRα1-4) (14).

RET homodimerization, mediated by a ligand-co-receptor complex, leads to the activation of several downstream pathways that include RAS/MAPK, PI3K/AKT, JAK/STAT, PKA, and PKC pathways. Thus, activation of RET signaling ultimately promotes cell proliferation, growth, and survival (15).

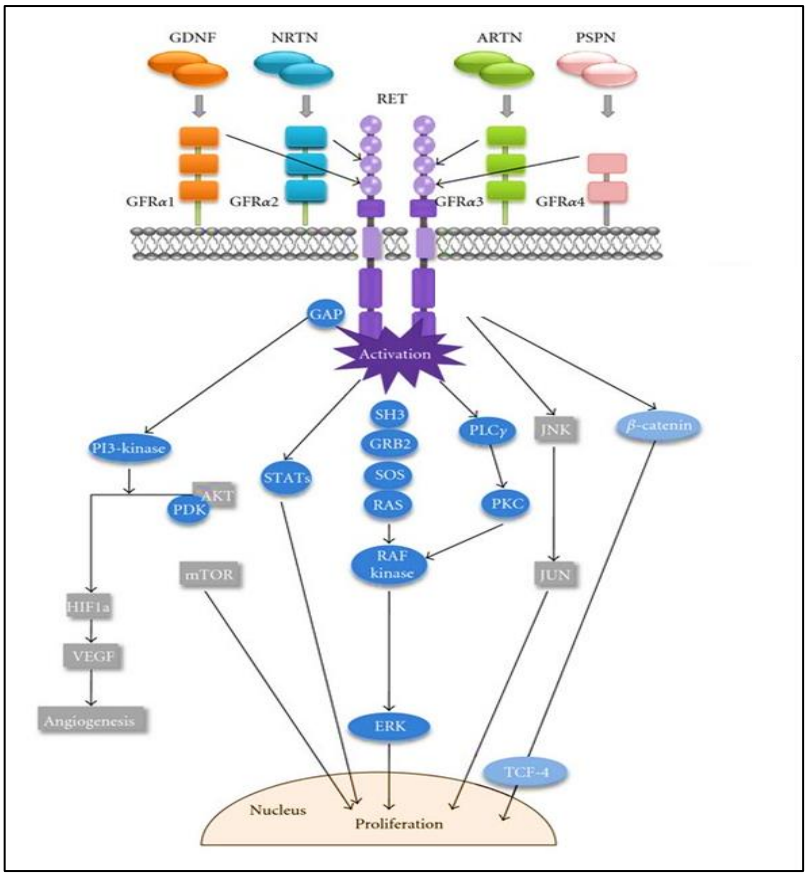


Fig.3: RET pathway activation signaling: Binding of GDNF-family ligands (GFLs) and GDNF to co-receptor GFRα1-4 induces recruitment of RET, forming RET-GFRα complex. Formation of the RET-GFRα complex brings two RET monomers near induce homodimerization and cross phosphorylation of key RET tyrosine residues that recruit adaptor proteins important for propagation of RET signaling, such as PI3K/AKT, MAPK, PLCγ, and RAS/RAF/ERK. Adopted by: Prazeres H, Torre J, et all. How to Treat a Signal? Current Basis for RET-Genotype-Oriented Choice of Kinase Inhibitors for the Treatment of Medullary Thyroid Cancer. Journal of Thyroid Research Vol. 2011, Article ID 678357, 10 pages doi:10.4061/2011/678357 (15).

RET mutations in medullary cancers

In MTC proto-oncogene RET exerts a crucial role in oncogenesis. Germline RET mutations are present in >98% of all familial cases of MTC and in 44% of sporadic MTC (10). In MTC, the RET gene typically carries point mutations, instead deletions or insertions are rare. The activating point mutations of RET may affect both extracellular and intracellular domains and as a result, induce different effects: extracellular mutation domains induce a ret activation, which is both

ligand and dimerization independent; furthermore, intracellular mutations domain induce a ligand-independent constitutive dimerization, promoting the activation of the tyrosine kinase receptor (16). Cysteine mutations in RET cysteine-rich domain promote the formation of intermolecular disulfide bridges. These bridges lead to the constitutive dimerization and activation of RET that is GDNF-independent. Mutations in tyrosine kinase domains can elicit steric conformations that regulate access to the ATP binding pocket of RET (E768X, V804X), alter hinge or inter-lobe flexibility (L790X, Y791X), promote activation of RET monomers (S891X, M918T), or destabilize the inactive form of RET (A883X), all of which confer constitutive RET activation albeit with varying activities (17). One of the most common and more aggressive mutations out of more than 60 activating *RET* mutations discovered is the RET^{M918T}. RET^{M918T} is located in the kinase domain and is characterized by higher ATP-binding affinity and altered *RET* autophosphorylation trajectory (18).

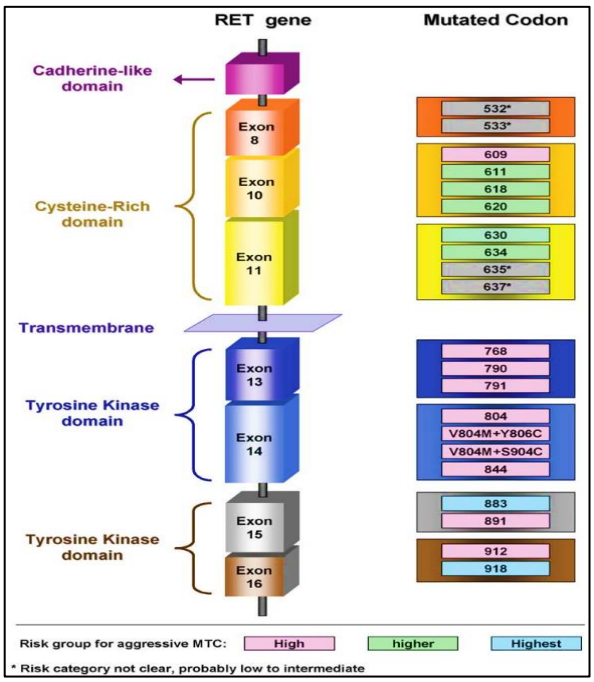


Fig.4: Representation of the RET gene and reported codons responsible for the three levels of biological aggressiveness of MTC. Adopted by: Kouvaraki MA, Shapiro SE, Perrier ND, Cote GJ, Gagel RF, Hoff AO, Sherman SI, Lee JE, Evans DB. RET proto-oncogene: a review and update of genotype-phenotype correlations in hereditary medullary thyroid cancer and associated endocrine tumors. Thyroid. 2005 Jun;15(6):531-44. doi: 10.1089/thy.2005.15.531. PMID: 16029119 (17).

Treating RET-altered thyroid cancers

The primary strategy of therapy for patients with MTC is surgery. Surgery, cytotoxic chemotherapy, targeted therapy, radiation therapy, endocrine therapy and immunotherapy are the current major treatments for cancer management. RET-altered thyroid cancer was treated with MultiKinase Inhibitors (MKIs) and/or with RET-specific inhibitors, a TyrosineKinase Inhibitors (TKI). One of the strategies to target RET is the utilization of MKI, in 2011 Cabozantinib and Vandetanib were approved by the U.S. Food and Drugs Administration (FDA) and the European Medicines Agency (EMA) as a treatment for inoperable progressive MTC or metastatic patients. Notably, Vandetanib gave rise to a greater objective response rate in M918T-mutant MTC (19). RXDX-105 has more potent RET protein kinase inhibitor activity than other RET MKIs, like Cabozantinib and Vandetanib, though, RXDX-105 was unable to inhibit RETV804M/L gatekeeper mutants. As a consequence, tumors with RETV804M progressed on RXDX-105 treatment. More recently the U.S. FDA proceeded with the approval of two RET-specific gatekeeper mutant-effective, Pralsetinib and Selpercatinib for the treatment of thyroid cancers with RET mutations. Pralsetinib (BLU-667) and Selpercatinib (LOXO-292) are an orally bioavailable selective RET inhibitors (20). They are more potent than other MKI RETs and inhibit RETV804M/L gatekeeper mutants. There have been identified acquired RET secondary mutations that cause resistance to specific RET TKIs in both preclinical experiments and in patients whose tumors acquired TKI resistance (21). The gatekeeper RETV804M/L mutants are resistant to Vandetanib, Cabozantinib, Lenvatinib, and other MKIs used to inhibit RET.

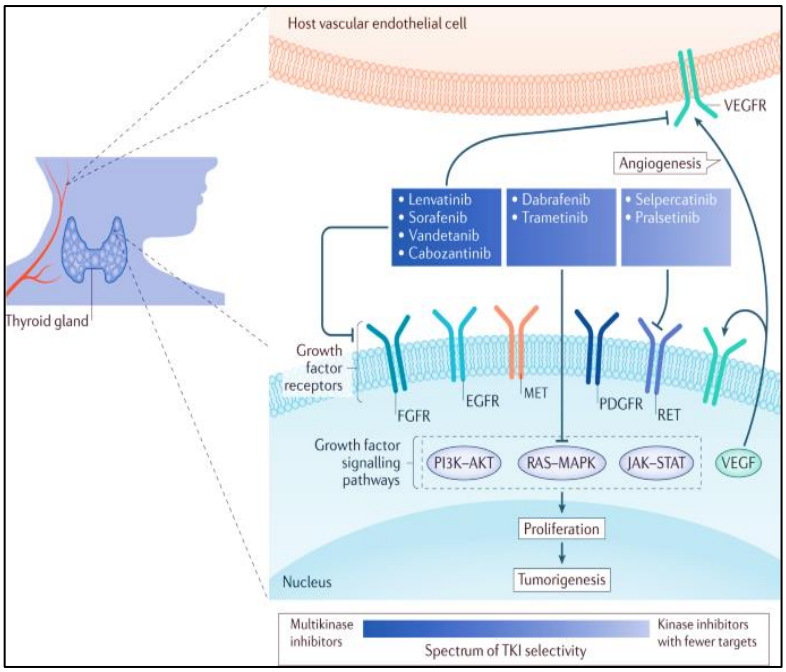


Fig.5: An overview of MTC therapy: Drugs and target, MKIs versus highly selective inhibitors. Adopted by: Gild ML, Tsang VHM, Clifton-Bligh RJ. et all. Multikinase inhibitors in thyroid cancer: timing of targeted therapy. Nat Rev Endocrinol **17**, 225–234 (2021). https://doi.org/10.1038/s41574-020-00465-y (21).

Polyphenols

Polyphenols are a large group of at least 10.000 different compounds that contain one or more aromatic rings with one or more hydroxyl groups attached to them. These are natural compounds found mostly in fruits (apples, cranberries, pomegranate juice), green and black tea, coffee, red wine, cocoa, and seed (22). These organic agents fall into several subclasses such as catechins, flavonoids (which contain flavonols, flavanols, and flavones), anthocyanins, catechins, isoflavones, chalcones, curcuminoids, and phenolic acid (23). Polyphenols, as anticancer agents, have many advantages such as high accessibility, low toxicity, specificity of the response and other various biological effects. The idea of using polyphenols for treating cancer patients is not new (24). The main advantage of polyphenols as anticarcinogenic agents is the combination of cytoprotective effects towards normal cells and cytotoxic effects towards cancerous (25). Furthermore, the fact that they attack exclusively cancer cells with a variety of mechanisms directed against tumor markers, makes these compounds beneficial and interesting (25). Polyphenols have been implicated as potential preventive

and curative therapeutic agents for a host of diseases like Alzheimer's, Parkinson's, Diabetes, Stroke, Autism etc. (26).

Antioxidant effects

Polyphenols act as antioxidants through several mechanisms. Among these mechanisms is building a barrier against oxidants generation or scavenging free radicals. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the main free radicals that exist in our cells and cause oxidative stress. Quercetin and Chrysin are confirmed to inhibit oxidants generation (27).

Pro-apoptosis effects

In cancer, anti-apoptotic pathways are activated. This involves the uncontrolled proliferation of cells, which leads to drug resistance and tumor recurrence. Cell death by apoptosis through the expression of apoptosis-related genes can be induced by a great number of polyphenols (28). Resveratrol increases apoptosis in many types of cancers including bladder, prostate, breast and ovarian. Evidence shows i.e. that the treatment of cells with Resveratrol has led to an increase in the activation and expression of caspase-3 and AMPK while reducing both activation and expression of mTOR and downstream kinase of AMPK. It has been shown that i.e. Quercetin induces apoptosis in methotrexate-resistant osteosarcoma cell line U2-OS/ MTX300 as evidenced by fluorescence staining and cytometry via mitochondrial dysfunction and dephosphorylation of Akt (29).

Epigenetic effects

Polyphenols help with the prevention of epigenetic dysregulation as DNA methylation, histone modifications and chromatin/nucleosome remodeling which are involved in a variety of cancer features (30). Curcumin is one of the most efficient polyphenols that prevent these alterations from aiding the cancer cells by inhibiting histone deacetylases (HDACs) and histone acetyltransferases (HATs) (31). Moreover, curcumin suppresses DNA methylation in the promotor region of many cancer-related genes, including the tumor suppressor gene as i.e. Wnt inhibitory factor-1, Nrf2 and RARβ2 by decreasing the DNA methyltransferase 1 level (DNMT1) (32).

Polyphenols and chemosensitization: Cancer drug resistance effects

There are several mechanisms that can cause drug resistance-MultiDrug Resistance (MDR) in cancer cells, such as increased efflux through trans-

membrane drug transporters. P-gp, MRP1, and BCRP, have been found to mediate MDR as drug efflux pumps. In response to chemotherapeutic agents, P-gp, MRP1, BCRP and at least 15 human ABC proteins, are upregulated, which, in turn, leads to a reduction in the drugs intracellular accumulation and decreases their efficacy (33). Polyphenols change the sensitivity of cancer cells to chemotherapeutic drugs, and this named chemosensitizers. Since the discovery of the first chemosensitizers, a wide variety of natural compounds have been documented for their ability to induce cell death by many molecular targets. Curcumin, Resveratrol and Epigallocatechin-3-gallate (EGCG), over-coming cancer drug resistance (34). There are several mechanisms through polyphenols that affect the sensitivity of cancer cells to chemotherapeutic agents such as drug uptake, drug efflux, drug metabolism and cell death. It was found that the expression level of the MRP1 gene in DOX-resistant acute myeloid leukaemia (AML)-2/DX300 cells, is higher compared to the wild-type AML-2/WT cells (35). Treating this chemoresistant cell line with Resveratrol has led to a reduction in MRP1 expression. Furthermore, Resveratrol is reported to enhance the absorption of the MRP1 substrate 5(6)-carboxyfluorescein diacetate. Therefore, it is suggested that Resveratrol may enhance Doxorubicin cellular absorption by reducing the gene expression of MRP1 (36). EGCG is shown to modulate P-gp function and enhance the intracellular entrapment of Doxorubicin in drug-resistant KB-A1 cells. As a matter of fact, the combination of Doxorubicin with EGCG has increased the Doxorubicin intracellular concentration by 2.3 times in KB-A1 cells in comparison to the treatment with Doxorubicin alone (25). Polyphenols have suppressed some enzymes involved in phase I and phase II of the drug metabolism. There have been reports that Curcumin may reduce the first-pass metabolism of Tamoxifen in the small intestine and/or in the liver. This is achieved by the inhibition of the CYP3A4 subfamily, a member of the cytochrome P450 family of oxidizing enzymes (37). Polyphenols induce apoptosis in cancer cells. It has been shown that i.e. Quercetin induces apoptosis in methotrexate-resistant osteosarcoma cell line U2-OS/ MTX300 as evidenced by fluorescence staining and cytometry via mitochondrial dysfunction and dephosphorylation of Akt (29).

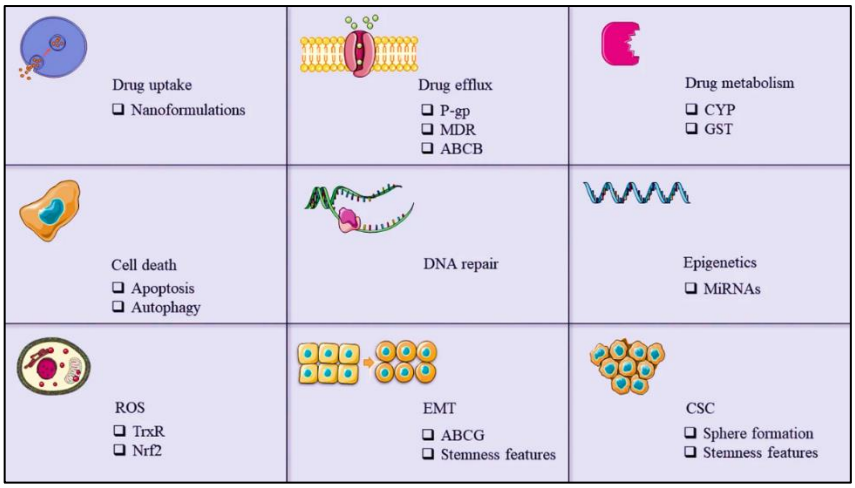


Fig.6: Overcoming cancer drug resistance: schematic representation of the mechanisms adopted by polyphenols in chemosensitization. Adopted by: Vinod BS, Maliekal TT, et all. Phytochemicals As Chemosensitizers: From Molecular Mechanism to Clinical Significance. VOL.18, NO.11 Comprehensive Invited Reviews normal Published Online: 25 Feb 2013 https://doi.org/10.1089/ars.2012.4573 (36).

Combinational therapy

In recent years, in order to treat devastating diseases such as cancer, many different combined therapeutic approaches have been used. Since tumors are almost always multiclonal and genetically heterogeneous, combinational therapies are strongly preferred. Due to drug resistance, therapeutic strategies that use single drugs are more likely to lead to treatment failure since the treatment kills sensitive cancer cells while resistant cancer cells are left to survive and proliferate. In comparison, a combinational therapy with the use of two or more drugs is more likely to target multiple driver genes simultaneously and not only inhibiting more clones in a tumor. This method though is also making new cancer mutations resistant to multi-drug treatment much more difficult to be selected and grow up. A recent trend of successful target drug therapy is the simultaneous use of multi-targeting drugs (38).

Moreover, combinational therapy aims to achieve a synergistic therapeutic effect, reduce the dose and toxicity, and minimize or delay the induction of drug resistance (39). After the study of several drug-phytochemical interactions, almost all interactions between pharmaceutical drugs and dietary polyphenols showed an increase in the therapeutic effects. This was achieved by blocking one or more targets of the signal transduction pathways, increasing the bioavailability of the

other drug or, stabilizing the other drug in the system (40). Many in *vitro* and in *vivo* studies suggest that when combined with chemotherapeutic agents (i.e. Doxorubicin, Cisplatin and Taxol), polyphenols can be used as chemosensitizers (41). Polyphenols act as chemosensitizing agents on many molecular targets, they are involved in cell cycle regulation, apoptosis, oxidation and angiogenesis. Some polyphenols interact beneficially when administered in combination, and synergistic chemosensitization properties of polyphenols are well known. Over the last few years, there have been reported several important and clinically relevant interactions between polyphenols and conventional drugs (42).

In *in vitro* studies, the Combination Index (CI) method is a quantitative representation of the pharmacologic interaction between two drugs. The median-drug effect analysis was originally described by Chou and Talalay and is the most widely used method in the literature for analyzing combinations. The program Calcusyn (now replaced by Compusyn) provides the software for quantifying synergism or antagonism (43).

OBJECTIVES

Co-occurrence of driver oncogenes, *de novo* and acquired resistance to MKIs or RET TKIs represent a main challenge to overcome in the treatment of tumors.

The aim of this project is to generate *in vitro* MTC-cells resistant to Vandetanib named TT resistant cell, evaluate some biological characteristics of this population and analyze the secondary resistance mechanisms, and investigate whether molecules belonging to the polyphenol class and Doxorubicin could sensitize Vandetanib-resistant MTC cell line.

Medullary thyroid cancer (MTC) cell line TT was chronically exposed to increasing concentrations of Vandetanib in order to generate MTC-resistant cells to Vandetanib. Once the TT resistant cell line was obtained, gatekeeper mutations *V804 (M/L)*, cell cycle distribution, cell proliferation, doubling time, colony-forming ability, Calcitonin release and apoptosis were studied. Some gene involved in cell cycle regulation, Epithelial-Mesenchymal Transition (EMT), stemness, and multidrug resistance (MDR) were studied. Subsequently, ABCG2 and P-gP protein were detected and *in vitro* chemosensitization with the Combination Index Method was performed. The combined cytotoxic effect of Pterostilbene, DMU212, Doxorubicin, Fisetin and Quercetin with Vandetanib on TT resistant cells were investigated.

TT resistant cell line does not acquire *V804 (M/L)* mutations, and reveled a G0G1 cell cycle arrest, a slowing cell proliferation, an inhibited colony-forming ability, an increased doubling time and ability to release calcitonin then TT cell line. Cytometric analysis for apoptosis showed an increased percentage of cell death in TT cells treated with Vandetanib. ABCG2 and P-gP protein detection by citofluorimetric analysis and western blotting, reveal increased protein expression. Evaluating the role of P-gP by cytometric analysis demonstrate that in TT resistant cell the pump does not have a remarkable activity.

The combined cytotoxic effect of Pterostilbene, DMU212, Doxorubicin, Fisetin and Quercetin with Vandetanib on TT-resistant cells shows that cytotoxicity is increased when Vandetanib is used in combination rather than when used alone, only for certain concentrations of the combinations of drugs tested. The study of the expression of some genes confirms that TT-resistant cells are a cell with a slower proliferation rate, the ability to exclude exogenous and endogenous toxic

substances, a lower colony-forming capacity, and a high ability to resist Vandetanib even in the presence of chemosensitizers.

MATERIALS AND METHODS

Compounds

Vandetanib (Selleckchem, MI, Italy) was dissolved as a 10 mM stock solution; Pterostilbene (Sigma Aldrich, MI, Italy) was dissolved as a 50 mM stock solution; Fisetin (Sigma Aldrich, MI, Italy) was dissolved as a 10 mM; Quercetin (Sigma Aldrich, MI, Italy) was dissolved as a 100 mM; EGCG [(-)-epigallocatechin-3-gallate] (Sigma Aldrich, MI, Italy) was dissolved as a 100 mM stock solution; DMU-212 (Sigma Aldrich, MI, Italy) (a methylated derivative of Resveratrol) was dissolved as a 20 mM stock solution. Doxorubicin (Sigma Aldrich, MI, Italy) was dissolved as a 1mM stock solution. All compounds were dissolved in Dimethyl Sulfoxide **(**DMSO) (Sigma Aldrich, MI, Italy) and stored at -80°C until use, then diluted with a medium at the appropriate concentration.

Cell culture

TT cell line is a stabilized cell line derived from human medullary thyroid carcinoma, and harbors a cysteine 634 to tryptophan (C634W) exon 11 RET mutation (44). TT cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured at 37°C in a humidified 5% CO2 in coated flasks with 0.01% calf skin collagen (Sigma Aldrich, MI, Italy) in F-12 Medium (Sigma Aldrich, MI, Italy) supplemented with 20% fetal bovine serum (EUROCLONE, MI, Italy), 2 mM L-glutamine and 100 units/ml penicillin-streptomycin (GIBCO, MI, Italy). TT cells were passaged once a week at a 1:2 split ratio.

Generation of TT resistant cells

In order to generate MTC resistant cells, TT cells were chronically exposed to increasing concentrations of Vandetanib. Starting from a dose of 10 nM, the drugs were added every 3 passages up to 0.5 μM. In this way, we isolated a mass population of TT cells adapted (named TT resistant cells) to proliferate at this Vandetanib dose. Resistant cells were cultured in Expansion Medium (EM), represented by a culture medium enriched with 500nM of Vandetanib.

Determination of IC50 value by MTT assay

Cell viability was evaluated using the MTT assay (Sigma Aldrich, MI, Italy). Cellular vitality has been expressed as a percentage to control cells. Briefly, TT cells and TT resistant cells were plated in 96 wells (CORNING, Arizona, U.S.) at

a density of $1X10^4$ cells per well in 100 µl culture complete medium in triplicate and exposed to increasing concentrations of Vandetanib, Pterostilbene, Fisetin, Quercetin, EGCG, DMU-212, Doxorubicin (from 0.390625 to 100 µM), and Verapamil (from 0.390625 to 200 µM), doubling the concentration for each condition and cultured for 3 days. Moreover, TT cells and TT resistant cells were cultured with Vandetanib for 3, 6 and 10 days. At the end of the time point, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed (Cell Proliferation Kit (MTT) Sigma Aldrich, MI, Italy). IC50 was calculated with a nonlinear fit equation curve by GraphPad Prism version 5.00 for Windows (GraphPad Software).

Isolation of total DNA and exon sequencing

Total DNA was extracted using the commercial PureLink® Genomic DNA Kit (Invitrogen, Thermo Fisher Scientific, Italy) following the manufacturer's instructions. DNA quantity and quality were evaluated by NanoDrop 2000 (Thermo Fisher Scientific, Milan, Italy). The exons 11 and 14 of the RET gene were amplified by conventional PCR utilizing the specific pair of primers presented in Table 1. Amplicons were detected using a 2% agarose gel and the fragment size was estimated using a DNA molecular weight marker (TrackIt™ 100 bp DNA Ladder, Invitrogen, Thermo Fisher Scientific, Italy). Amplicons were purified by QIAquick Gel Extraction Kit (Qiagen, Germany), quantified and marked using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Italy). Sequencing reactions before capillary electrophoresis were purified with BigDye Xterminator TM Purification Kit (Thermo Fisher Scientific, Italy). Exon sequencing was performed using the 3500 Genetic Analyzer (Applied Biosystems, USA) with polymer POP-7 as a separation matrix. Nucleotide sequence identity compared with reference with GenBank database using the Basic Local Alignment Search Tool (BLAST) and aligned using SnapGene.

Cell cycle analysis

TT cells and TT resistant cells were plated at a density of $5X10^5$ in T25 wells (CORNING, Arizona, U.S.) in culture complete medium, with or without Vandetanib at 500 nM concentration, respectively. After 6 days, the cells were harvested and Cold Spring Harb Protoc was assessed (45). Briefly, $5X10^5$ cell resuspended in 1 ml of ice-cold DPBS w/o Ca_2+/Mg_2+ (EUROCLONE, MI, Italy). Following fixation in ice-cold 100% ethanol (Sigma Aldrich, MI, Italy),

cells were resuspended in 500 µl of ice-cold cell cycle buffer (30 µg/ml propidium iodide, 100 µg/ml DNase-free RNase A in DPBS w/o Ca_2+/Mg_2+) (Sigma Aldrich, MI, Italy). All samples were then acquired using a FACSCalibur flow cytometer (Becton-Dickinson, New Jersey, USA) and analyzed by the ModFit software (Verity House).

Cell proliferation curve and Population Doubling Time (DT)

The proliferation rate of TT cells and TT-resistant cells was evaluated by trypan blue assay (Sigma Aldrich, Milan, Italy). TT cells and TT-resistant cells were seeded in a 24-well plate (CORNING, Arizona, U.S.) at a density of $1.8X10^3$ cell/well in 1ml culture complete medium and grown up to 480 hours. Every 72 hours, the cells were harvested and stained with trypan blue and counted under optical microscopy. Cell proliferation curve was obtained with the exponential growth equation by using GraphPad Prism version 5.00 for Windows (GraphPad Software). The DT was calculated according to the literature data (46).

Colony Forming Unit (CFU) assay

TT cells and TT-resistant cells were seeded in 10 cm^2 dishes at a density of 500, 300, and 100 cells/dish, and cultured under opportune conditions, refreshing old medium every 3 days. After 46 days, Crystal Violet Assay was performed. The colonies were counted by optical microscope observation. Only cellular groups containing more than 50 cells were considered as colonies. Three sets of experiments for each sample were performed for calculations.

Calcitonin assay

To measure calcitonin release TT cells and TT-resistant cells were plated at a density of $5X10^5$ in T25 wells (CORNING, Arizona, U.S.) in a culture complete medium. The day before the assay the medium was changed. On the day of the assay, the medium was collected and centrifuged at maximum speed, and an aliquot of 100 µl was used to perform the Human Calcitonin ELISA assay (Sigma-Aldrich, Milan, Italy). The amount of Calcitonin detected will be expressed in pg/mL normalized by the number of cells.

Determination of Apoptosis

To detect apoptosis in TT cells and in TT resistant cells after exposure to Vandetanib, cytofluorimetric analysis was used to quantify cell numbers in apoptosis. Briefly, TT cells and TT resistant cells were plated at a density of

$5X10^5$ in T25 wells (CORNING, Arizona, U.S.) in culture complete medium with Vandetanib at 100 nM concentration. After 24 hours cells were tripsinized and analyzed by cytometry analysis.

Isolation of total RNA and quantitative Real-Time PCR (qRT-PCR)

Isolation and purification of total RNA was performed using the RNeasy Mini Kit (Qiagen, California, USA), according to the manufacturer's instructions. RNA quantity and quality were evaluated by NanoDrop 2000 (Thermo Fisher Scientific, Milan, Italy); 2 μg of TT cells and TT-resistant cells of RNA were reverse-transcribed to cDNA in a volume of 20μl with Oligo dT primers using the QuantiTect Reverse Transcription Kit (Qiagen, California, USA). Quantitative PCR (qPCR) was performed using the QuantiNova SYBR Green PCR Kit and the RotorGene Q Instrument (Qiagen, California, USA). The cDNA samples were mixed with the SYBR Green PCR master mix and a specific pair of primers is presented in Table 2. The qPCR assay was performed according to the Qiagen instruction. The specificity of the amplified products was determined by the melting peak analysis. The relative expression of target genes was calculated using the ΔΔCt method according to Livak et al. (47). β-actin was used to normalize the expression of target genes.

Flow cytometric analysis

TT cells and TT-resistant cells were plated at a density of $5X10^5$ in T25 wells (CORNING, Arizona, U.S.) in culture complete medium, with or without Vandetanib at 500 nM concentration, respectively. Afterwards, cells were harvested and washed in ice-cold DPBS w/o Ca_2+/Mg_2+-BSA 1%. (EUROCLONE, MI, Italy) (Sigma Aldrich, MI, Italy). The cells were stained with human anti-ABCG2 antibody (mouse monoclonal IgG2b, anti-human ABCG2, Santa-Cruz Biotechnology, diluted 1:20). All samples were then acquired using a FACSCalibur flow cytometer (Becton-Dickinson, New Jersey, USA) and analyzed by CellQuest Pro software.

Doxorubicin accumulation by cytofluorimetric analysis

TT cells and TT resistant cells ($3X10^5$/ml) were incubated with or without Verapamil at a concentration of 50 μM, for 24 hours, then treated with Doxorubicin at a concentration of 2, 4 and 6μM for 2 hours. The cells were then washed, trypsinized and washed twice with cold PBS (CORNING, Arizona, U.S.). The fluorescence intensity of cells was determined by the flow cytometry

(FACSAria III; Becton Dickinson, San Jose, CA, USA). Doxorubicin has an intrinsic fluorescence with an emission signal at 595 nm upon excitation with a 488 nm laser. The effect of Verapamil on cell growth was assayed by the MTT method, as previously described. 50µM concentration of Verapamil has no cytotoxic effect on resistant TT and TT resistant cells (MTT date not show). All samples were analyzed by BD FACSDiva (Becton-Dickinson, San Jose, CA, USA).

Isolation of total protein and western blott analysis

Whole-cell extracts were prepared from TT cells and TT resistant cells. The cells were lysed in 1X RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Italy) and quantified by Bradford assay (Thermo Fisher Scientific, Italy). 20 µg total proteins were separated in SDS-PAGE (7% polyacrylamide gel) (Thermo Fisher Scientific, Italy) and transferred onto a nitrocellulose membrane (Amersham Biosciences, Amersham, United Kingdom). Membranes were blocked in blocking solution (TBS-milk5%) (MERCK, Germany), and probed with primary antibodies over-night at 4°C (ABCG2 (D5V2K) XP® Rabbit mAb or MDR1/(E1Y7B) Rabbit mAb Cell Signaling Techonology). The primary antibodies were detected with a polyclonal goat anti-rabbit immunoglobilin conjugated with HRP (Anti-Rabbit IgG (H+L), or Anti-Mouse IgG (H+L), PROMEGA, Milan, Italy) for 1h at room temperature. Signals were developed with SuperSignal™ West Femto Maximum Sensitivity Substrate Super Signal (Thermo Fisher Scientific, Italy) in Molecular Imager® ChemiDoc™ XRS System (Bio-Rad Italy). The intensity of western blotting strength was evaluated by ImageJ software.

Determination of Combination Index (CI) value by MTT assay

Combination Index (CI) was evaluated using MTT assay (Sigma Aldrich, MI, Italy). TT-resistant cells were exposed to combinations in a Constant Ratio of Vandetanib and polyphenols (Pterostilbene, DMU212) or Doxorubicin at IC50 and IC50x0.25, IC50x0.50, IC50x2; IC50x4 doses and cultured for 3 days. At the end of the time point, the MTT assay was performed (Cell Proliferation Kit (MTT) Sigma Aldrich, MI, Italy). The combined effects of different concentrations of polyphenols with Vandetanib were evaluated, and the combination index (CI) values were determined using CompuSyn software. CompuSyn program is based on the median-effect equation, derived from the mass-action law principle, which

is the unified theory that provides the common link between a single entity and multiple entities, and first-order and higher-order dynamics. This general equation encompasses the Michaelis-Menten, Hill, Henderson-Hasselbalch and Scatchard equations in biochemistry and biophysics.

Statistical analysis

All the experiments of the study were performed in triplicate, and results are reported as means ± SD and compared by the Student's unpaired two-sample T-test. $P \leq 0.05$ was considered statistically significant.

RESULTS

Selection and Morphologically features of TT resistant cells

Chronic exposure to increasing doses of Vandetanib (starting dose from 10 nM to 0.5 μM), after approximately 5 months, generated MTC-resistant cells TT to Vandetanib. TT-resistant cells were more refractile, spindle-shaped and staked than parental cells. Overall, the cells grew in clusters and were less adherent to the culture flask (Fig.7).

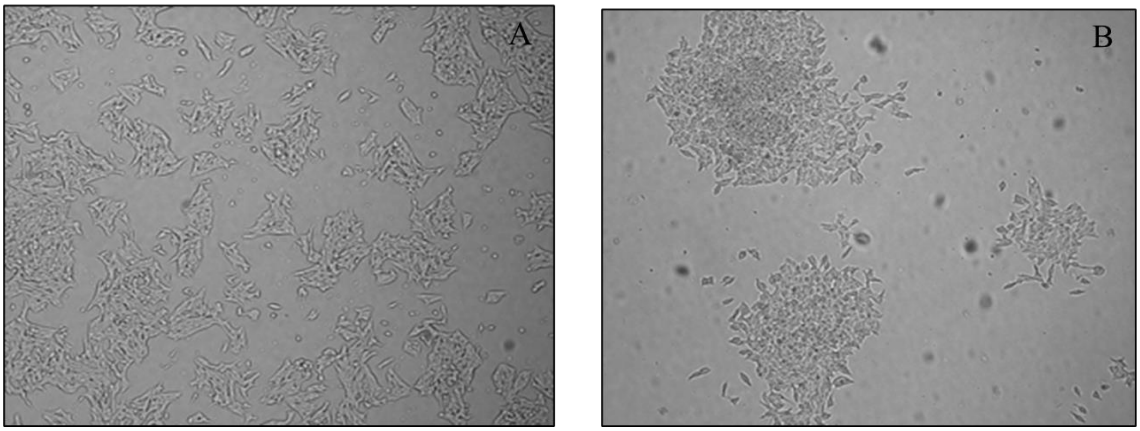


Fig.7: TT cells (A), and TT resistant cells (B) were photographed with an optical microscope at 5X magnification in a bright field.

TT resistant cells-IC50 evaluation

IC50 value for Vandetanib in TT cells was estimated to be 467±20 nM, 300.7±32 nM, 275.2 ±17 nM, at 3, 6 and 10 days respectively, while in TT resistant cells was 1108±57 nM, 794.1±68.5 nM, 928.6±70 nM at 3, 6 and 10 days respectively (Fig.8). As expected from the establishment of resistance, the IC50 value in TT resistant cells compared to TT cells is ~3-fold higher at 3, 6, and 10 days, respectively.

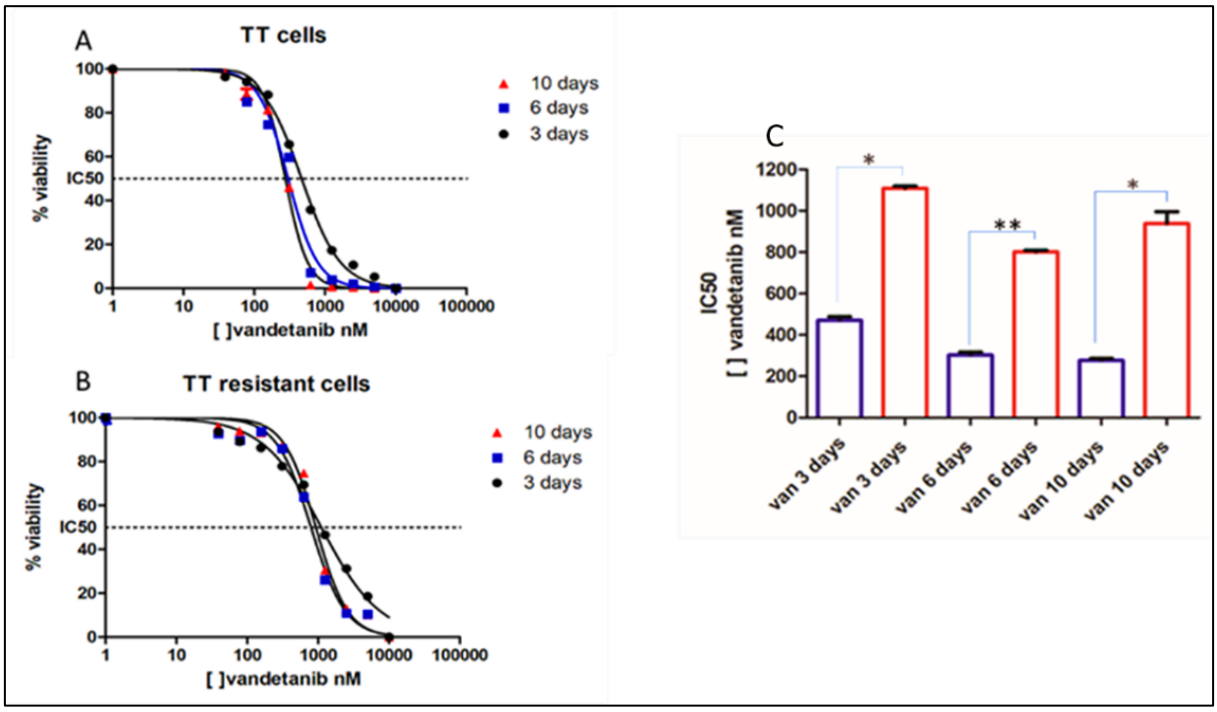


Fig.8: Inhibition curve of Vandetanib on TT cells and TT resistant cells. The dose-response inhibition curve of TT cells (A) and TT resistant cells (B) by Vandetanib. The histogram bar represents the IC50 value in TT cells (blue) and TT resistant cells (red) at 3, 6, and 10 days (C). The data was presented by mean±SD of three replicates. p-values *p≤0.05, **p≤0.01.

Exon Sequencing

In many studies (20) it was reported that the secondary resistance to Vandetanib was due to gatekeeper mutations at the codon *V804 (M/L)* (exon 14 gene *RET*). In order to clarify whether the TT resistant cells retained this mutation, targeted Sanger sequencing was performed. It emerged that the TT resistant cell line did not acquire c.47480G>R - p.Val804Met/Leu in *RET* gene (Fig.9). Upon alignment, the region of exon 14 sequenced show 100% homology with TT cells. Moreover, TT cells and TT resistant cells conserved *C634(R)* (exon 11 gene *RET*) mutation which is the mutation characterizing this cell line (date non show).

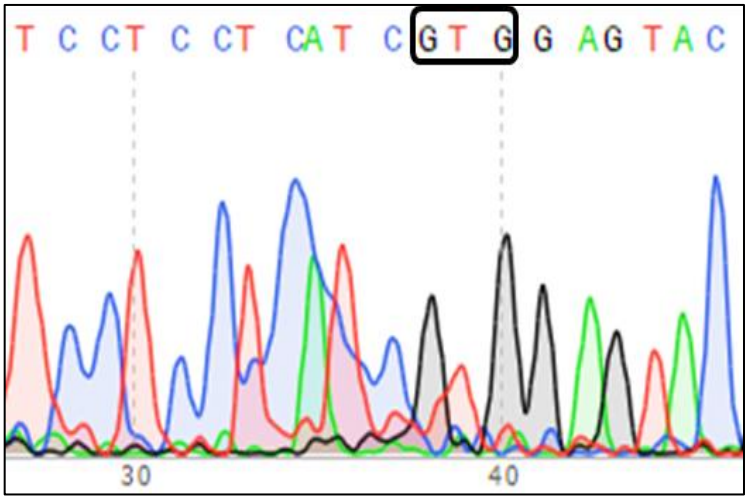
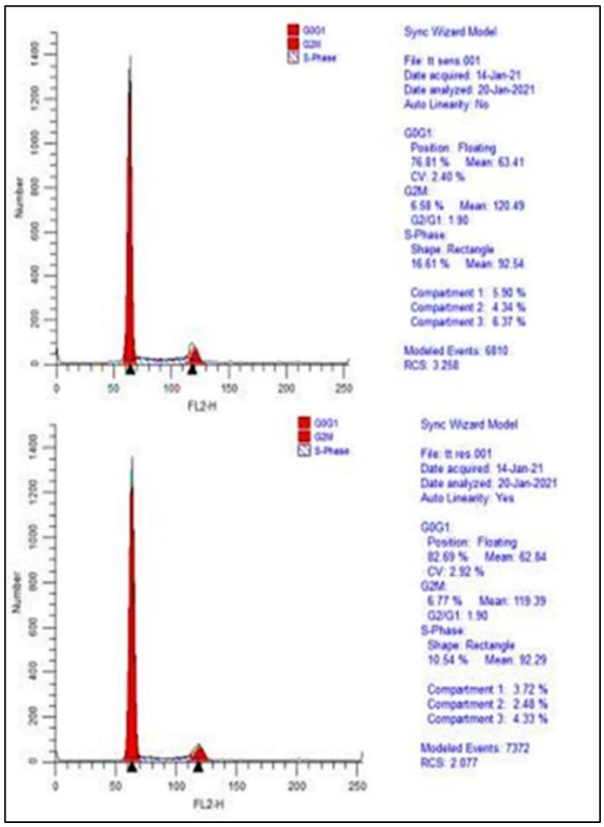


Fig.9: Exon sequencing. The images represent electropherogram of exon 14 that shown the triplet leading to *RET V804 (M/L)* mutation, TT resistant cell line carries wild type triplet (indicated by rectangle). The data presented is representative of three replicates.

Cell cycle distribution

To examine the possibility that Vandetanib resistance may modify the cell cycle, the cell cycle of TT cells and TT resistant cells was investigated. It was observed that the resistance to Vandetanib in TT resistant cells increased the cell population in G0/G1 phase (5.8±0.9% p≤0.05) and decreased cell number in S-phase (6.04±0.027 p≤0.0001) (Fig.10) suggesting a cell cycle arrest in G0/G1.



	TT CELL	TT RESISTANT CELL
G0G1	76,81% *p≤0.05	82,69% *p≤0.05
G2M	6,58%	6,77%
S	16,61% **p≤0.01	10,54% **p≤0.01

Fig.10: Cell cycle analysis of TT cells and TT resistant cells. Cells cycle and the percentage of TT cells and TT resistant cells in the different cell cycle phases, as represented by ModFit software, are indicated in the left panel. A representative table of the distribution in the cell cycle of TT cells and TT resistant cells is reported in the right panel. The data presented is representative of three replicates. p-values.*p≤0.05, **p≤0.01.

Cell proliferation and Doubling Time

Cell proliferation is decreased in TT-resistant cells rather than in TT cells as shown in the cell growth curve (Fig.11A). As it is highlighted in the cell growth curve, after 72h, TT cells started to proliferate faster than TT-resistant cells, showing a higher proliferation rate. The calculated doubling time (DT) was 136.5±5h vs. 158.7±8h in TT cells and TT resistant cells, respectively (Fig.11B).

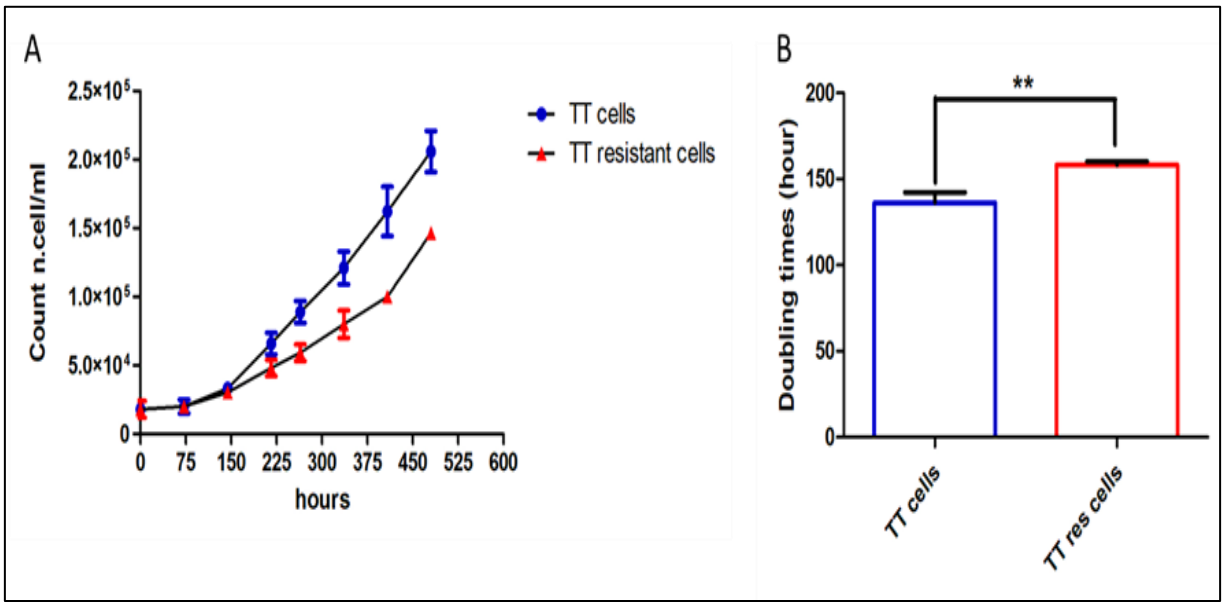


Fig.11: Cell growth analysis. Figure (A) shows the cell growth curve of TT cells and TT-resistant cells. Figure (B) shows the DT of TT cells and TT resistant cells. The data was presented by mean±SD of three replicates. p-values **p≤0.01.

Colony-forming assay

The effect of Vandetanib resistance and the capacity of cells to proliferate and produce colonies was determined by colony-forming assay. As shown in Figure 12, Vandetanib significantly inhibited the colony-forming ability in TT-resistant cells compared to the TT cells. The counting performed by software ImageJ showed 85.6 ± 2.1 (500 cells/dish), 50 ± 4.2 (300 cells/dish) and 10 ± 0.9 (100 cells/dish) for TT cells, 11.5 ± 3 (500 cells/dish), 6 ± 1.3 (300 cells/dish) and 3.2 ± 0.73 for TT resistant cells.

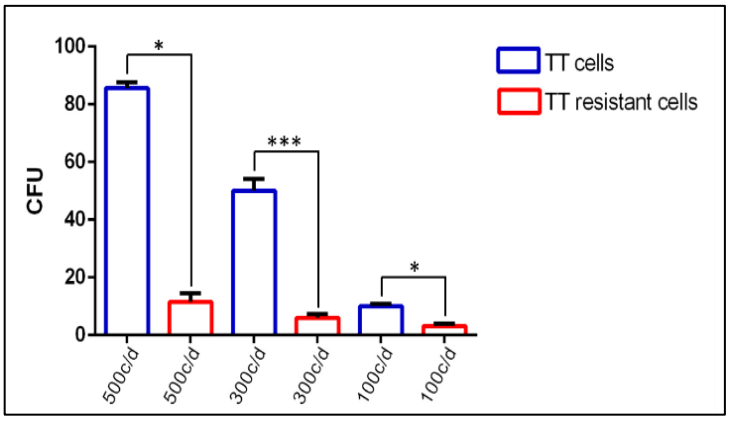


Fig.12: Colony formation ability. The histogram bar represents the number of colonies produced by cells plate with decreasing confluence. CFU: Colony Formation Unit. The data are reported as mean values ± SD of three independent experiments. p-value *P≤0.05; ***P≤0.001.

Apoptosis induction

To determine whether Vandetanib-induced cell death by apoptosis citometric analysis was performed. Results revealed an increased percentage of cells death (2 v/s 8.2 %) in TT cells treated with Vandetanib at 100 nM concentration. In TT resistant cells Vandetanib treatment does not have any apoptotic effect.

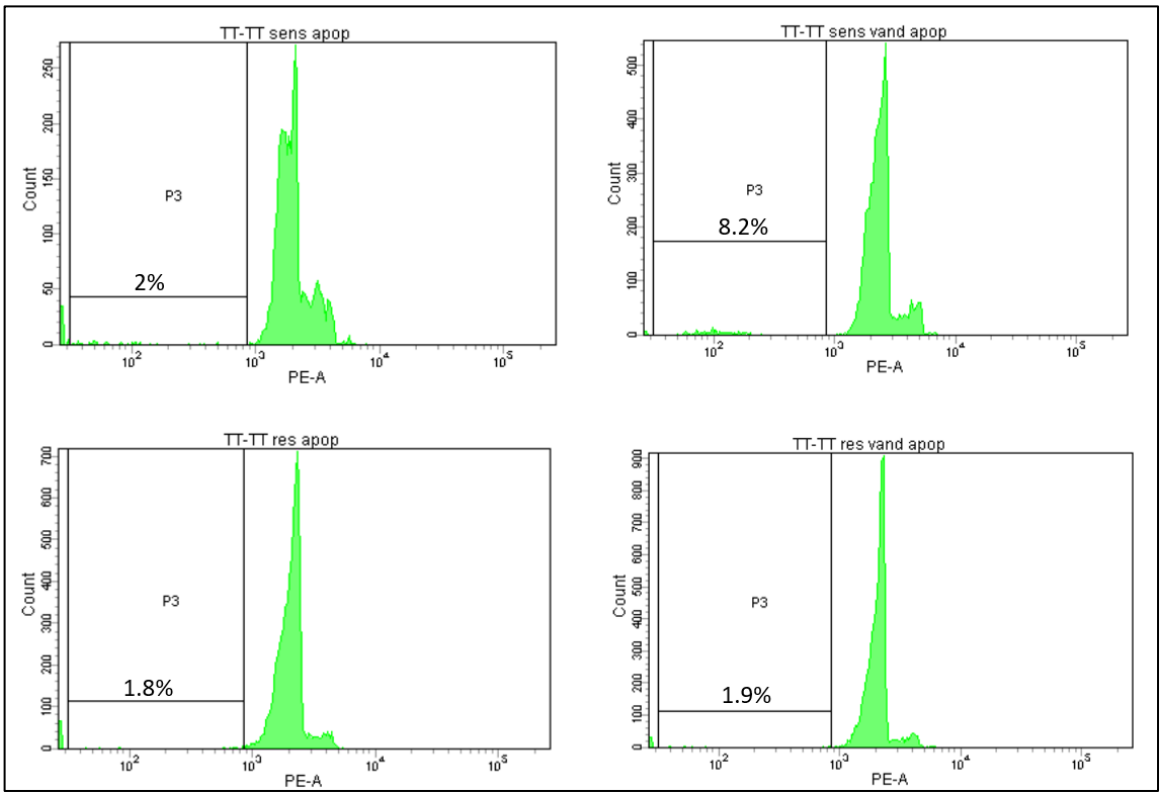


Fig.13: Apoptosis analysis. Histograms plot of TT cells (top panel) and TT resistant cells (bottom panel) after incubation with Vandetanib (right panel) or without Vandetanib (left panel). The data presented is representative of three replicates.

Cytometric detection of ABCG2 protein

To study the mechanism of resistance, the expression of ABCG2 protein in TT resistant cells compared to TT cells was evaluated. Anti-ABCG2 antibody revealed an increase of positivity of about 6% in TT-resistant cells with respect to TT cells (Fig.14).

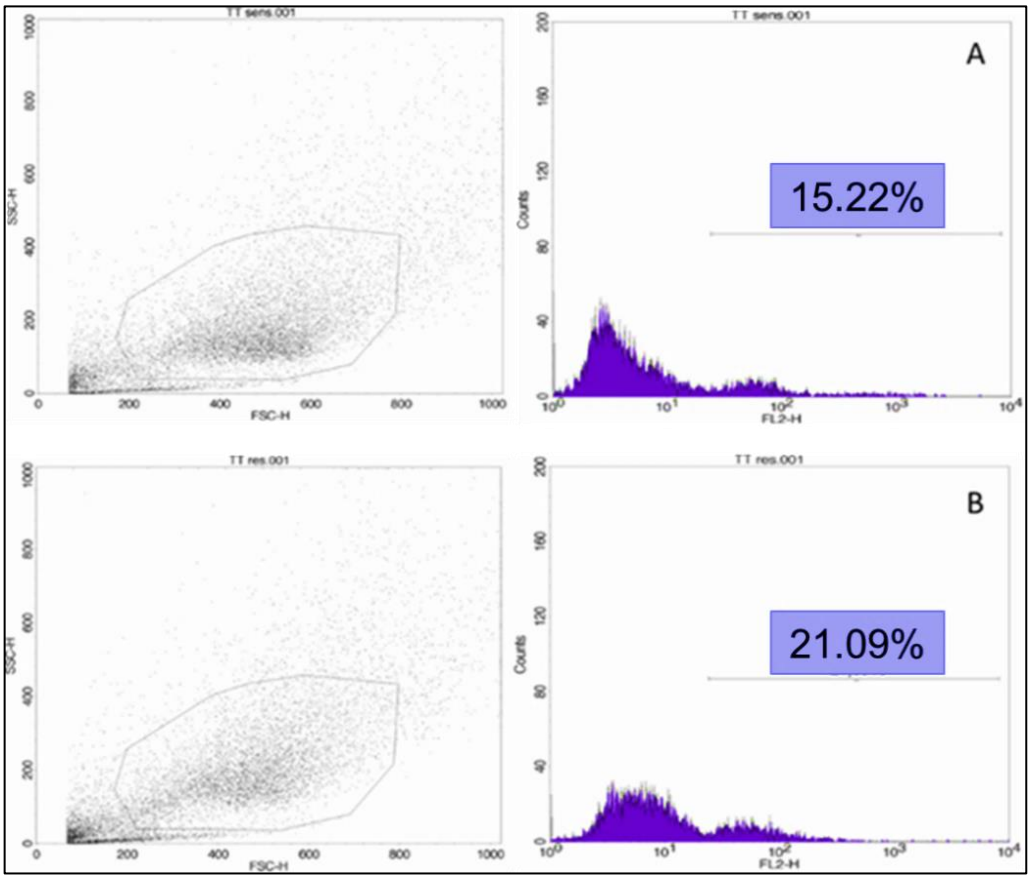


Fig.14: Flow-cytometric analysis of ABCG2. Histograms plot of ABCG2 expression in TT cells (A) and in TT resistant cells (B). Isotype control is not shown. The data presented is representative of three replicates.

ABCG2 and P-gP expression analysis by western blotting

To examine ABCG2 and P-glycoprotein expression levels in TT cells and in TT resistant cell lines immunoblotting was performed. TT resistant cells had the highest ABCG2 and P-glycoprotein expression levels compared to TT cells. The ABCG2 and P-glycoprotein expression level in these cells was positively correlated with their drug resistance.

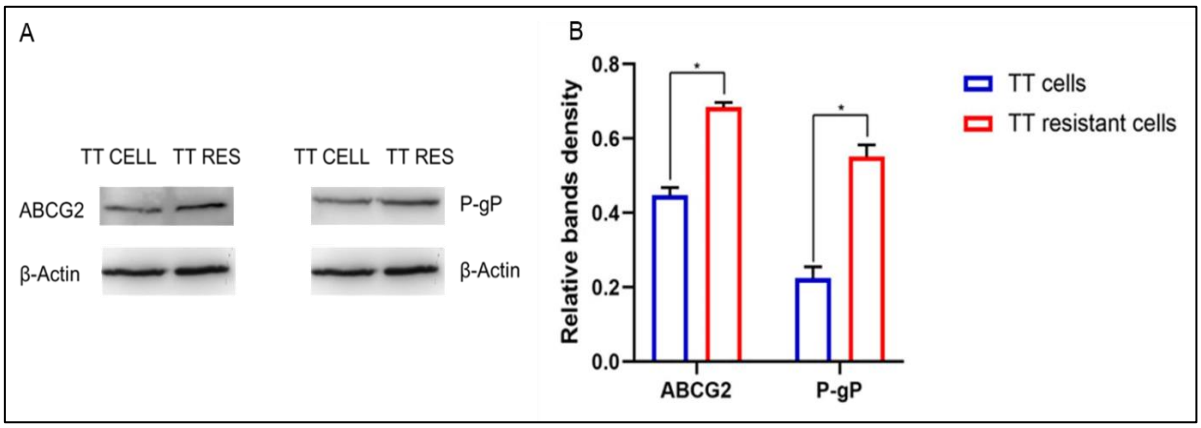
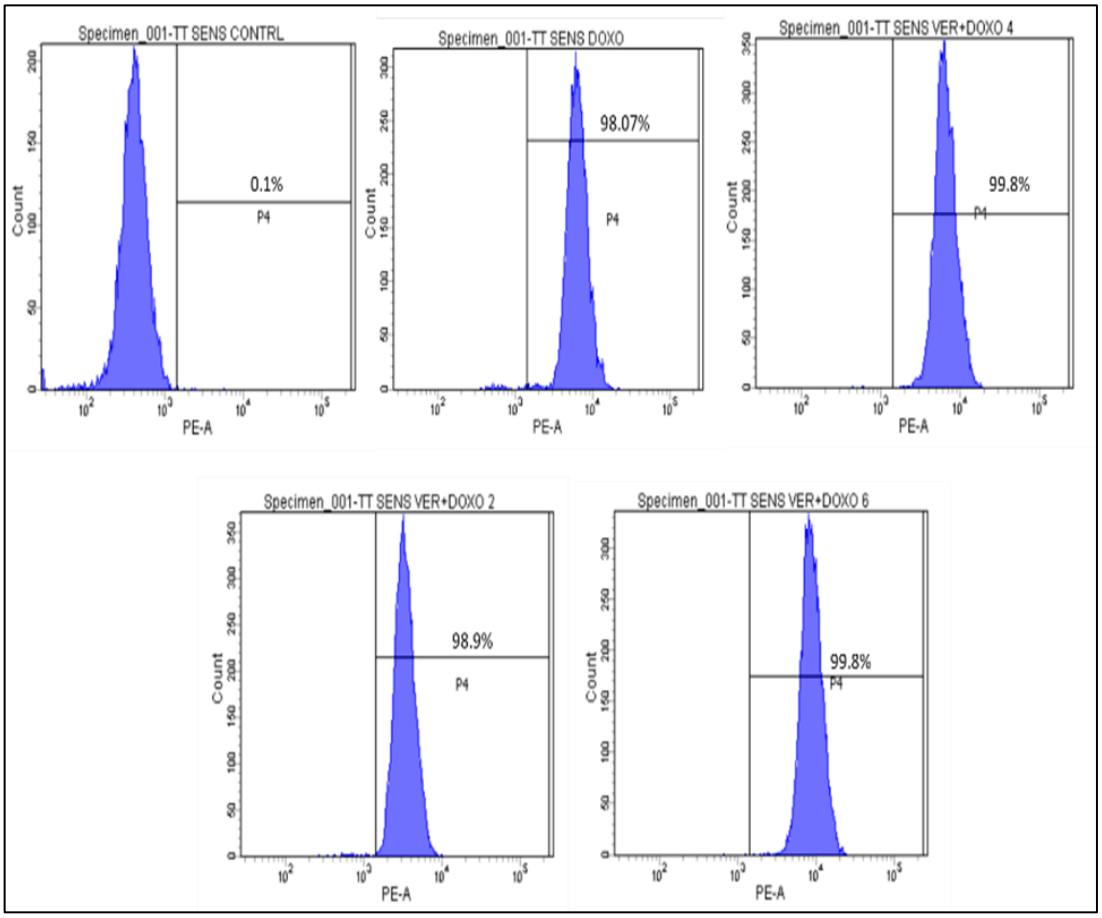


Fig.15: Western blotting analysis on TT cells and TT resistant cells to detected ABCG2 and P-glycoprotein. (A) Western blotting bands for ABCG2 and P-gP are reported. β-actin is presented as a loading control. (B) Representation of western blotting bands quantifications. The data was presented by mean±SD of three replicates. p-values *$p \leq 0.05$.

Combined treatment to study Vandetanib resistance by cytofluorimetric assay- Doxorubicin accumulation

A method for evaluating the role of P-gP in TT resistant cells is based on the decreased efflux of a fluorescent P-gP substrate, such as Doxorubicin, and in an increased accumulation in presence of Verapamil, a P-gP inhibitor. TT cells and TT resistant were treated with Doxorubicin (2 µM for 2 hours) or Verapamil (50 µM for 24 hours) and Doxorubicin (2/4/6 µM for 2 hours). In TT cells and in TT resistant cells, the Doxorubicin accumulation is essentially the same for all concentrations, demonstrating that in cell lines studied the absence of remarkable activity of pump activity.



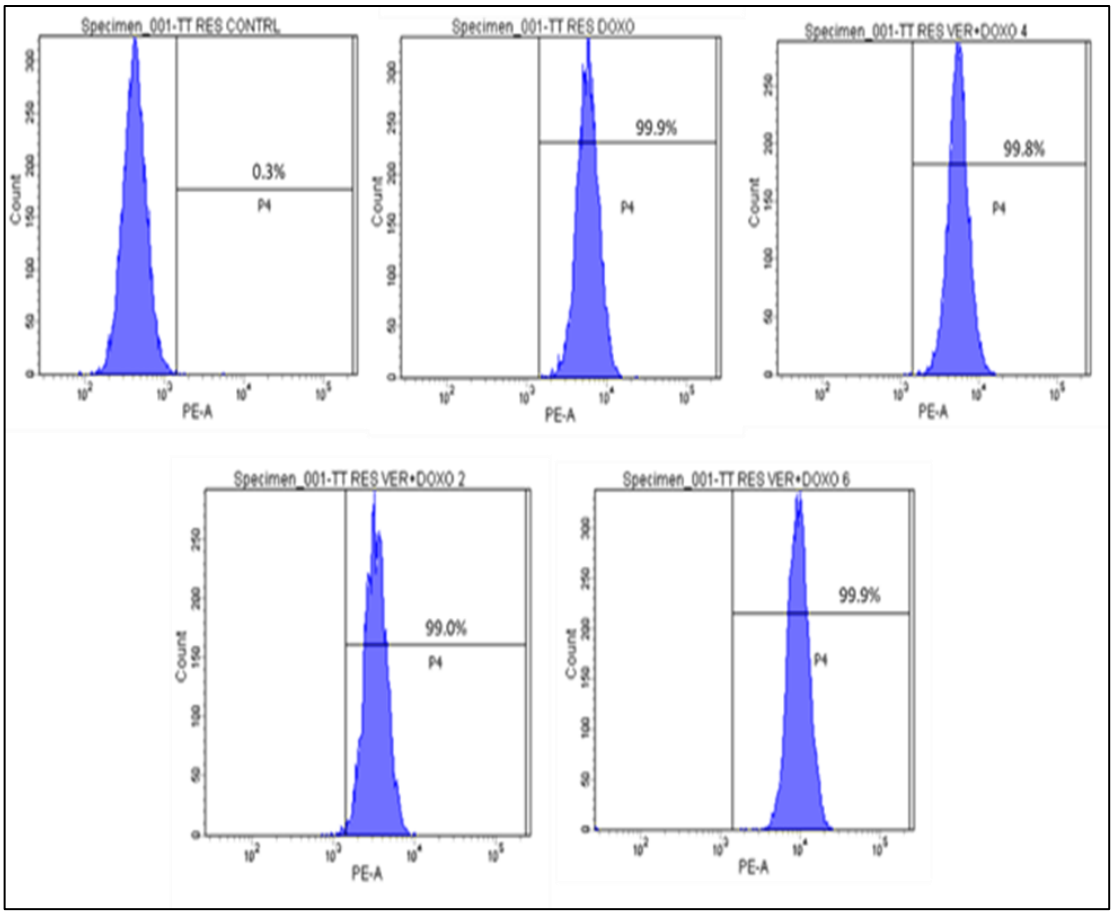
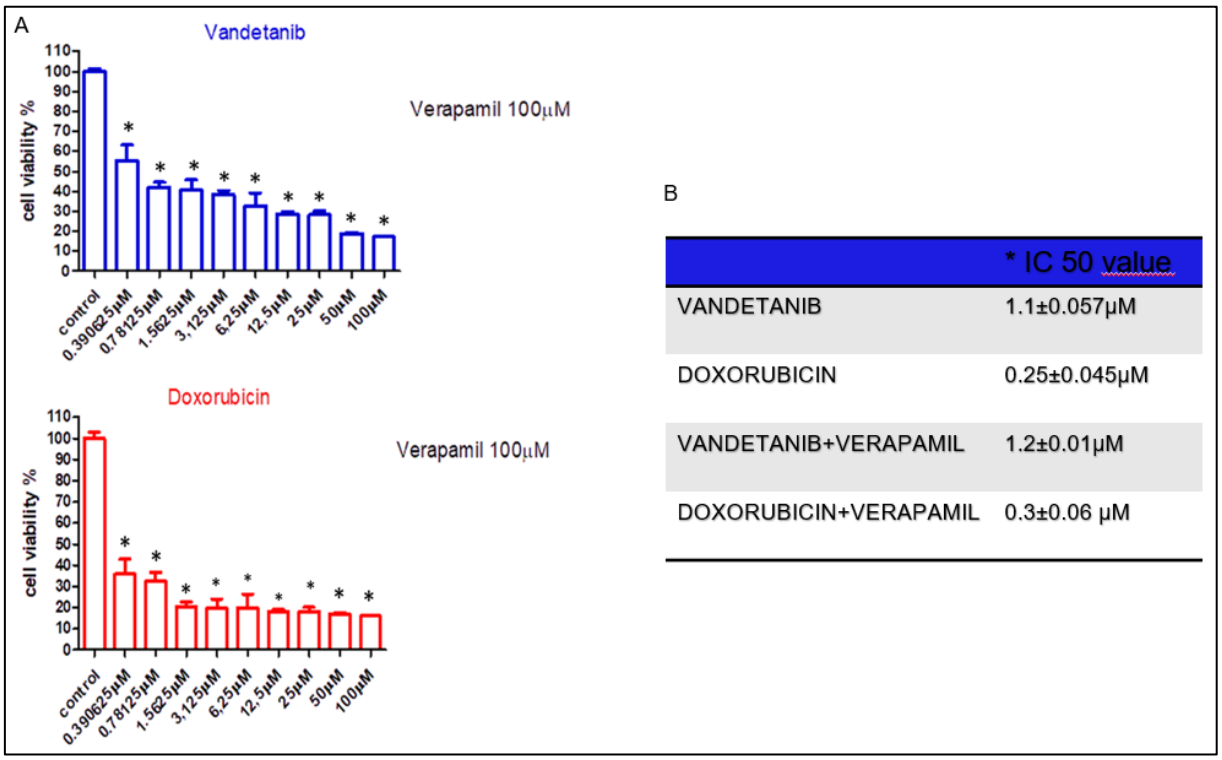


Fig.16: Doxorubicin accumulation: Histograms plot of TT cells (top panel) and TT resistant cells (bottom panel) incubate with Doxorubicin or Doxorubicin and Verapamil. The data presented is representative of three replicates.

Combined treatment to study Vandetanib resistance by MTT assay

TT cells and TT resistant cells were treated with Doxorubicin or Vandetanib at decreasing concentrations (from 100 µM to 0.390625 µM) in the presence of Verapamil (a specific P-gp inhibitor) at a constant concentration of 100 µM and then MTT was performed. As shown in Fig.17, a significant reduction in cell viability was observed after co-treatment with Doxorubicin and Verapamil or Vandetanib and Verapamil for all concentrations. However, the values of IC50 for Vandetanib and Doxorubicin alone or in combination with Verapamil reveal that the cytotoxic effect of drugs in the presence of the P-gP inhibitor pump is comparable to the cytotoxic effect of Vandetanib and Doxorubicin alone. The same values of IC50 were calculated.



	* IC 50 value
VANDETANIB	1.1±0.057µM
DOXORUBICIN	0.25±0.045µM
VANDETANIB+VERAPAMIL	1.2±0.01µM
DOXORUBICIN+VERAPAMIL	0.3±0.06 µM

Fig.17: Effects of combined treatment with Verapamil and Vandetanib or Doxorubicin on TT resistant cells. (A) The histogram bar represents cell viability of TT resistant cells. (B) The table shows the IC50 values measured. The data was presented by mean±SD of three replicates. p-values *p≤0.05.

Gene expression analysis

In order to investigate the behavior of TT resistant cells in terms of cell cycle distribution, Epithelial-Mesenchymal Transition (EMT), multidrug resistance, transcription factors and stemness, some genes representative of these characteristics and correlated with the expression of RET, were detected.

To clarify the molecular meaning of the cell distribution in the cell cycle, CCDN1 and its inhibitor CCDNK1 expression was evaluated. The expression of CCDN1 was found to decrease by about 46% whereas, as expected, the expression of its inhibitor increased by about 61% in TT resistant cells. The expression of BCL2 was found to decrease by about 66% in TT resistant cells.

In TT resistant cells SNAIL, SLUG, MMP9, VIMENTIN were decreased by about 34%, about 27%, about 69%, about 79% respectively; instead TWIST was increased by about 36%.

Moreover, in TT resistant cells, the qRT-PCR analysis showed a down-expression of stem genes OCT3-4, C-MYC, and SOX2, of about 13%, about 53%, and about 33% respectively.

Finally, to investigate the molecular basis of Vandetanib resistance, the expression of ABCG2 and MDR-1 was analyzed. An increase of about 46% of MDR1 was detected in TT resistant cells compared to TT cells. Whereas an unchanged ABCG2 expression was found. RET gene is equally expressed in TT cells and TT resistant cells.

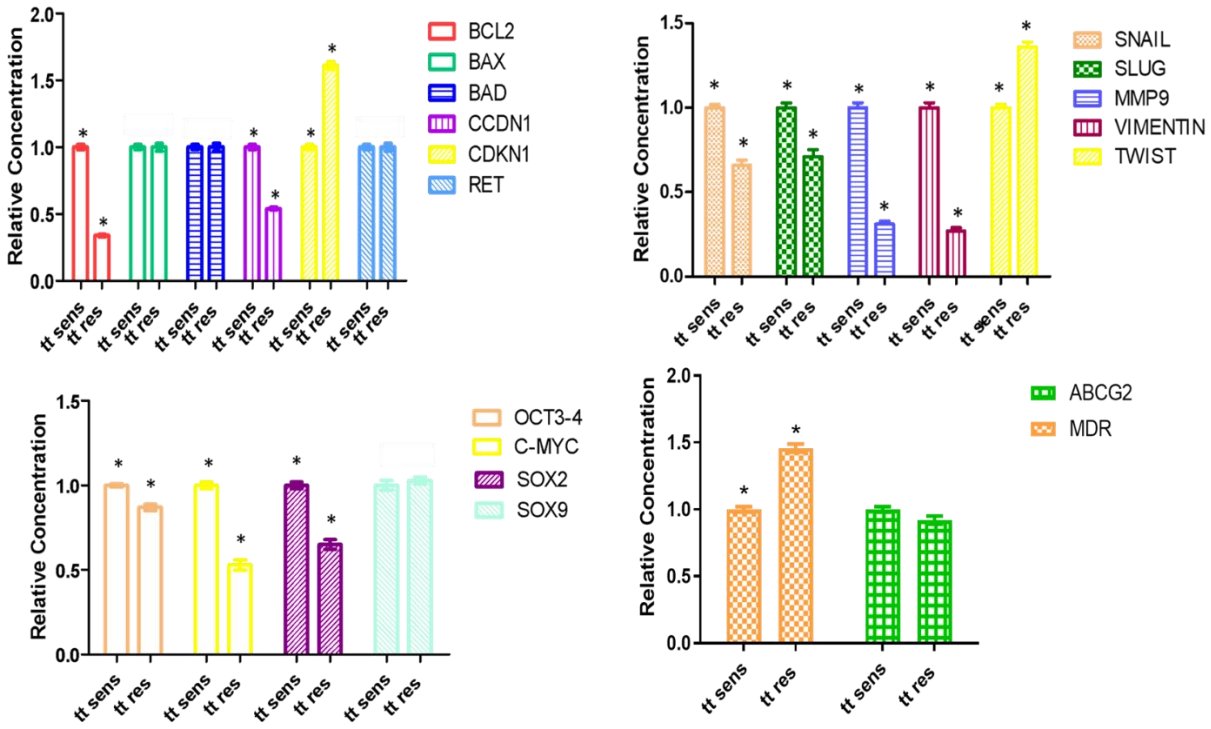


Fig.13: Gene expression overviews. Histograms show the expression of BAX, BAD, BCL2, CDKN1, CCDNK1, RET, SNAIL, SLUG, MMP9, VIMENTIN, TWIST, OCT3/4F, C-MYC, SOX2, SOX9, MDR, ABCG2 in TT cells and TT resistant cells. Actin-β was used as a housekeeping gene. The data was presented by mean±SD of three replicates * $p<0.05$.

Calcitonin Release

The capability to release calcitonin by TT cells and TT-resistant cells was analyzed. Calcitonin released by TT resistant cells was about four times higher than that one released by TT cells (210 pg/1X10^6 cell versus 788 pg/1X10^6 cell, $p \leq 0.05$).

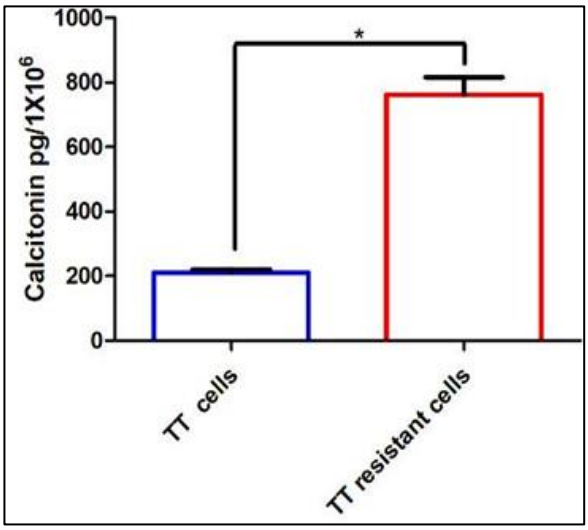


Fig.15: Calcitonin release. Histogram shows Calcitonin released from TT cells and TT resistant cells in the culture medium expressed in pg/cell. The data was presented by mean±SD of three independent experiments. *$p \leq 0.05$

Effects of Pterostilbene, Fisetin, Quercetin, EGCG, DMU212, Doxorubicin on TT resistant cells-IC50 evaluation

IC50 value for Pterostilbene, DMU212 and Doxorubicin was estimated to be 5±0.047 µM, 5.5±0.062 µM and 0.25±0.045 µM, respectively. Compared to the control, low toxicity was observed for Fisetin, Quercetin and EGCG, all of them tested up to 100 µM, making the calculation of the IC50 not possible (Table 3).

Table 3: IC50 values on TT-resistant cells incubated with various concentrations of Vandetanib, Doxorubicin and polyphenols: Pterostilbene, DMU212, Fisetin, Quercetin and EGCG

Drugs	***IC50 (µM)**
Pterostilbene	5±0.047
DMU212	5.5±0.062
Doxorubicin	0.25±0.045
Fisetin	>100
Quercetin	>100
EGCG	>100
Vandetanib	1±0.057

*IC50: Concentration that inhibited cell growth by 50%. The data was presented by mean±SD of three independent experiments, *p≤0.05.

Combined effects of Pterostilbene, DMU212, Doxorubicin, Fisetin and Quercetin with Vandetanib on TT resistant cells - Combination Index

The effects of different concentrations of polyphenols as Pterostilbene, DMU212 and Doxorubicin in combination with Vandetanib were evaluated for cytotoxicity. The combined cytotoxic effect was found to be lower or higher depending the concentrations used. For most drugs tested, cytotoxicity is lower when used in combination with Vandetanib than when used alone. This means that antagonism was found. The Combination Index (CI) was found to be lower when polyphenols, Doxorubicin and Vandetanib were used at the maximum concentrations tested (Table 4). Five different concentrations (ranging from 0.25x to 4x the IC50) of each polyphenol and Doxorubicin in combination with Vandetanib were evaluated. The results of the comparisons between 15 different groups are shown in Table 4. Strong antagonism effects were observed in most combinations of Vandetanib with drugs. A strong synergistic effect was detected when Vandetanib and Pterostilbene or DMU212 or Doxorubicin were combined at maximum doses (4xIC50). Synergism and slight synergism were detected when the Vandetanib was combined at IC50 concentration with DMU212 (5.5 µM) or Doxorubicin (0.25 µM) (Table 4).

Table 4: The combined effect of Pterostilbene, DMU212, and Doxorubicin with Vandetanib on TT resistant cells after 72h of incubation

Combination (µM)	**Cytotoxicity (%)**	**DRI**	**CI Value**	**Conclusion**
Van (0.25) + Ptero (1.25)	13.78±2.87	0.32;1.12	3.98	Strong Antagonism
Van (0.5) + Ptero (2.5)	24.77±3.09	0.55;3.99	2.03	Strong Antagonism
Van (1) + Ptero (5)	36.29±1.68	0.69;8.39	1.56	Strong Antagonism
Van (2) + Ptero (10)	1±1.58	0.0001;0.0002	10	Strong Antagonism
Van (4) + Ptero (20)	68.6±3.00	2.02;0.23	0.5	Synergism

Van (0.25) + DMU212 (1.375)	11±2.98	0.19;25	5.26	Strong Antagonism
Van (0.5) + DMU212 (2.75)	24±4.50	0.48;88	2.08	Strong Antagonism
Van (1) + DMU212 (5.5)	44.4±3.05	1.19;294	0.84	Slight Synergism
Van (2) + DMU212 (11)	51.1±5.25	0.94;255	1.05	Antagonism
Van (4) + DMU212 (22)	71.1±3.00	2.07; 734	0.48	Strong Synergism
Van (0.25) + Doxo (0.0625)	20±4,59	0.64;5.19	1.74	Strong Antagonism
Van (0.5) + Doxo (0.125)	31±1.98	0.88;6.67	1.28	Strong Antagonism
Van (1) + Doxo (0.25)	47±1.85	1.42;9.98	0.80	Slight Synergism
Van (2) + Doxo (0.50)	66±3.26	2.75;17.62	0.42	Strong Synergism
Van (4) + Doxo (1)	71±2.08	2.05;12.8	0.56	Strong Synergism

In this table, Combination Index (CI) values and Dose-Reduction Index (DRI) were obtained from the contents generated by the CompuSyn report. The CI was calculated using CI equation algorithms with CompuSyn software. CI=1, <1, and >1 indicate an additive effect, synergism, and antagonism, respectively. The DRI was calculated from the DRI equation and an algorithm using CompuSyn software. DRI=1, >1, and <1 indicate no dose reduction, favorable dose reduction, and unfavorable dose reduction, respectively, for each drug in the combination. Van: Vandetanib, Ptero: Pterostilbene, Doxo: Doxorubicin.

It was not possible to calculate the IC50 for Fisetin and Quercetin, as the toxicity observed was less than 50% at the maximum tested concentrations (100 µM). It proceeded by combining the first non-toxic concentration of the individual polyphenols (25µM) which was kept constant, with varying concentrations of Vandetanib. This chemotherapeutic was added starting from 0.390625 to 10µM, doubling the concentration for each condition. The concentration of 10 µM corresponds to 10 times the IC50 of Vandetanib at 72h in TT-resistant cells (1µM).

IC50 value for Vandetanib in combination with Fisetin was estimated to be 2.14±0.049 µM, while in combination with Quercetin IC50 was 2.153±0.071 µM. The IC50 value in both cases is approximately double compared to TT resistant cells treated only with Vandetanib. Demonstrating also, in this case, an antagonism between the drugs used which results in a reduction of their overall cytotoxic effect compared to if they were used individually.

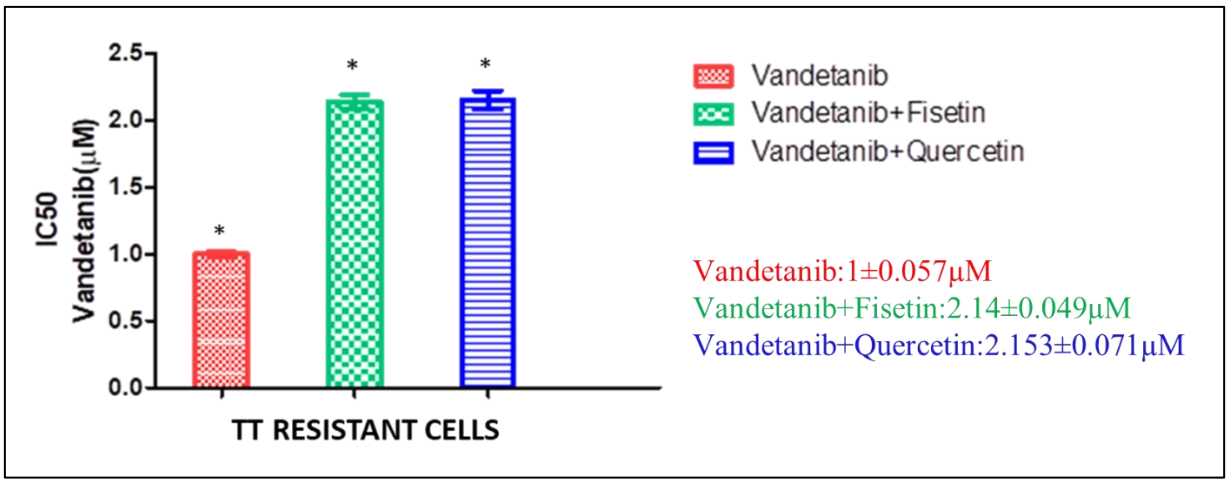


Fig.16: IC50 value for Vandetanib. The histogram bar represents the IC50 of Vandetanib alone and in combination with Fisetin or Quercetin in TT resistant cells. The data was presented by mean±SD of three replicates. p-values *p≤0.05.

DISCUSSION

To fight thyroid cancers there have been developed various therapies such as surgery, radioiodine therapy, and chemotherapy. In general, most thyroid cancers are treated surgically by removing the entire thyroid gland. When cancer cells migrate to the lymph node around the neck, the lymph node should also be removed. In addition, for the metastatic thyroid cancer, further therapy is required including radioiodine and hormone replacement therapy (48).

A well-known phenomenon in cancer is drug resistance. This occurs when cancer becomes tolerant to pharmaceutical treatment. Based on the time it was developed drug resistance can be categorized either as intrinsic or acquired. While intrinsic resistance already exists before drug treatment, the acquired resistance is induced after therapy, each occurring in about 50% of cancer patients. (39).

Many factors can contribute to drug resistance, such as genetic mutations and/or epigenetic changes, upregulated drug efflux, and various other cellular and molecular mechanisms (49).

Recent trends of successful target drug therapy have shown that simultaneous multi-targeting will be more effective in treating drug resistance. As a result, the anticancer efficacy of therapies is enhanced and the lives of the patients are prolonged. However, the outcome of therapies inevitably depends on the resistance profile of tumors and the toxicity tolerance of patients, with unpredictable therapeutic results (39).

Drug resistance developed in thyroid cancer patients treated with Multikinase inhibitors (MKIs) and/or recently Tyrosine Kinase Inhibitor (TKIs) is partially known. It is essential to understand the biological and/or pharmacological mechanisms to overcome the resistance in the future.

In this project, to study the Vandetanib resistance mechanism the MTC cells line TT was used as a model. Firstly, the TT cell line was chronically exposed to increasing concentrations of Vandetanib to generate MTC-resistant cells to Vandetanib. After 5 months, TT resistant cells were obtained. TT resistant cells were more refractile, spindle-shaped and staked than parental cells. To characterize the TT resistant cells, Sanger sequencing for gatekeeper mutation named *V804M/L* was performed. TT resistant cells do not acquire the *V804M* mutation but conserved the *C634R* mutation which is the characterizing mutation in the TT cell line. In addition, there have been evaluated other possible

mechanisms adopted by cells for escaping the toxic effect of Vandetanib. Cytometric cell cycle assay, cell proliferation assay and doubling time calculation were performed. The slowing down of proliferation rate, as indicated by G0/G1 cell cycle arrest, cell growth curve and the higher doubling time detected in TT resistant cells than in TT cells, is an indication of resistance. Taken together, these results suggest that TT resistant cells may have acquired an enhanced ability to prevent effects caused by Vandetanib. This, in turn, may have led to a reduced proliferation rate.

Finally, this property can be, also, due to overexpression of CCDNK1 and a down expression of CCND1.

Subsequently, there have been investigated main specific stem genes including SOX2 and OCT4 transcription factors. SOX2 and OCT4 were down-expressed. A colony formation assay to investigate the clonogenic property of the TT resistant cells was performed. TT resistant cells showed a much lower percentage of colony formation than TT cells, confirming what has been observed by the expression of stem factors.

Epithelial-mesenchymal transition (EMT) is known to play a critical role in the development and invasiveness of MTC. Through this process, tumor cells are known to acquire mesenchymal features and to become invasive, losing their epithelial characteristics (50). This change is accompanied by increased expression of a set of pleiotropically acting transcription factors (i.e. SNAIL, SLUG, VIMENTIN). In light of this, it is of great importance to investigate the expression of EMT-related genes. In TT resistant cells the expression of SNAIL, SLUG, MMP9 and VIMENTIN were downregulated than in TT cells. These results suggest that TT resistant cells are cells that retain their epithelial characteristics.

A possible mechanism of chemoresistance is the increased ability to exclude exogenous and endogenous toxic substances using the ATP-binding cassette (ABC) transporters. Among various ABC transporters, ABCG2 and P-gP are thought to have a critical role (51). Thus, to quantify the ABCG2 and MDR1 expression in TT-resistant cells qRT-PCR was assessed. Overexpression of MDR1 was detected whereas an equal ABCG2 mRNA level was found in TT resistant cells compared to TT cells. A cytofluorimetric analysis and western blotting showed an increased expression of ABCG2. An increased expression was

detected also for P-gP, but the evaluation of the activity of P-gP by cytometric analysis demonstrates that in TT resistant cells the pump does not have a remarkable activity than TT cells.

In short, the qRT-PCR results all confirm that TT-resistant cells are a cell with a slower proliferation rate, increased doubling time, a lower colony-forming capacity, a lower epithelial mesenchymal transition features and increased ability to exclude exogenous and endogenous toxic substances, than TT cells. Thus, these properties can be due to overexpression of CCDNK1, MDR, TWIST and a down expression of CCND1, SNAIL, SLUG, MMP9, VIMENTIN, OCT3-4, C-MYC, SOX2 in TT-resistant cells compared to TT cells.

MTC is characterized by high levels of calcitonin, which increases in metastatization and progression of the MTC (52). It was found that TT-resistant cells secreted higher levels of Calcitonin compared to TT cells. It can be speculated that induced chemoresistance to Vandetanib could be related to an alteration in the mechanism that regulates the production of Calcitonin by C cells.

Subsequently, the mechanisms responsible for the resistance were investigated by trying to re-sensitize the TT resistant cells to Vandetanib through the use of natural compounds (i.e. polyphenols), as well as other compounds.

With this objective in mind, TT resistant cells were exposed to combinations in a Constant Ratio of Vandetanib and Pterostilbene, or DMU212, or Doxuricin. Furthermore, because it was not possible to calculate the IC50 for Fisetin and Quercetin in TT resistant cells it was proceeded by combining the first non-toxic concentration of the polyphenol which was kept constant, with varying concentrations of Vandetanib.

The combined cytotoxic effect of Pterostilbene, DMU212, Doxorubicin, Fisetin and Quercetin with Vandetanib on TT resistant cells shows that cytotoxicity is increased when Vandetanib is used in combination, only for certain concentrations of the combinations of drugs tested. Demonstrating a synergism between the drugs used which results in an increase in their cytotoxic effect compared to if they were used individually.

CompuSyn Software based on Chou-Talalay Theory permits the investigation of drug combinations in *in vitro* studies. The Combination Index (CI) is a mathematical way to express pharmacologic drug interactions (43) where $CI \geq 1.1$ indicates antagonism, CI between 0.9 and 1.1 indicates additive effect, instead,

CI<0.9 indicates synergism. CI is shown to be the simplest possible way for quantifying synergism or antagonism and reducing the experimental size of animals used or the number of patients needed for drug combination clinical trials (53).

The C_{max} for Vandetanib in MTC patients following 300 mg daily dose (dose recommended by AstraZeneca) was 857 ng/mL (385-2241 ng/mL). Since the free drug in plasma is 6% of the total drug, the free drug concentration is about 51 ng/mL (≡ ~100 nM) (54). Unfortunately, the concentration of 100 nM is much lower than the concentration used in this work to achieve a synergistic effect.

Moreover, this study shows that the antagonistic effect of compounds such as Pterostilbene, DMU212 and Vandetanib is an important finding to be considered. The reduction of their effect when used in combination, compared to their individual use, demonstrates a relation between the two drugs and that genomic and proteomic profiling of tumors is crucial for strategically improving the therapeutic efficacy. Several pre-clinical studies including combinations of MKIs, TKI and polyphenol inhibitors have shown encouraging results and hold promise for further investigation in clinical trials (55).

A recent study (56) reports that P-gp activity increased after incubation for 72 hours of K562 cells with flavonoids and imatinib at high doses, resulting in an increased value of IC50 and CI. It was felt that this effect is related to the increased expression and activity of the proteins that transport substances out of cells, resulting in an increased P-gp activity. The cause of the increased P-gp may be due to the high doses and long incubation with drugs. After incubation with flavonoids and imatinib, the cells acquired resistance.

In TT resistant cells, it was observed that, after 72h of incubation, increasing concentrations of Vandetanib and polyphenols or Vandetanib and Doxorubicin give a greater synergistic effect with decreasing CI values. This suggests that after increased drug doses in TT resistant cells, there is no increase in P-gP activity and thus no additional resistance mechanism is established.

All this assumes even more importance given that, owing to inherent and acquired chemoresistance, a combination of different drugs is often used in preclinical and clinical trials to improve therapeutic efficacy.

Based on the results described in this study, chemoresistance can be reversed in MTC cells by treatment with Vandetanib in combination with polyphenols. The

results provide information on the resistance mechanism to Vandetanib in MTC cell line and give a new approach for the treatment of MTC in association with current therapies based on the use of TKI or MKI with polyphenols. This could extend the lifespan of MTC patients and improve their quality of life. However, further research is needed to elucidate the complete mechanisms involved in the polyphenols' chemosensitization.

Although polyphenols can enhance the chemosensitivity of the TT resistant cells to Vandetanib, it would be of clinical significance if the anti-MTC effects and the chemosensitization ability of polyphenols to revert Vandetanib-resistance should be confirmed *in vivo*. More studies must be conducted on this topic in the future.

In addition, evaluating drug treatment responses in primary cell cultures of patient tumors might help in guiding second-line treatment to develop precision medicine. The development of precision medicine will benefit from a comprehensive analysis of pharmacological markers to predict the course of treatment.

TABLES

Table 1: Primer sequence list for amplification of exons 11 and 14.

Exon	**Primer sequence**	**Brand/code number**
11	*F:5'CTCGATGGGGTGTTCTCAGG3'* *R:5'GGAGGGCAGGGGATCTTC3'*	Eurofins Genomics
14	*F:5'GCTCCTGGAAGACCCAGG3'* *R:5'TGGTGGAGTCAGGGTGTGACA3'*	Eurofins Genomics

F=forward; *R*=reverse

Table 2: Real-Time qPCR primer sequence list for amplification of TT cell and TT resistant cells cDNA.

Gene	**Primer sequence**	**Brand/code number**
β-actin	*F:5-'CCACACTGTGCCCATCTACG-3'* *R:5'-AGGATCTTCATGAGGTAGTCAGTCAG-3'*	EUROFINS GENOMICS
SOX2	*F:5'-GGAGACGGAGCTGAAGCCGC-3'* *R:5'-GACGCGGTCCGGGCTTGTTTT-3'*	MWG
SOX9	*F:5'-GACTTCTGAACGAGAGCGAGA-3'* *R:5'-CGTTCTTCACCGACTTCCTC-3'*	EUROFINS GENOMICS
OCT3/4F	*F:5'-GCTGGAACAGAAGAGGATCACC-3'* *R:5'-TACAGAACCACACTCGAACCAC -3'*	MWG
C-MYC	*F:5'-TCCTCGGATTCTCTGCTCTC-3'* *R:5'-CTCTGACCTTTTGCCAGGAG-3'*	EUROFINS GENOMICS

SSEA-4	*F:5'-TGGACGGGCACAACTTCATC-3'* *R:5'-GGGCAGGTTCTTGGCACTCT-3'*	EUROGENTEC
NFKB1	*F:5'-ATGGCTTCTATGAGGCTGAG-3'* *R:5'-GTTGTTGTTGGTCTGGATGC-3'*	EUROFINS GENOMICS
CDKN1B_1_SG	*QT00998445*	QUANTITECT PRIMER ASSSAY
CCNDN1_1_SG	*QT00495285*	QUANTITECT PRIMER ASSSAY
BAX	*QT00031192*	QUANTITECT PRIMER ASSSAY
BAD	*QT00997353*	QUANTITECT PRIMER ASSSAY
SNAIL	*F:5'-GCTCCTTCGTCCTTCTCCTC-3'* *R:5'-CCTTTCGAGCCTGGAGATCC-3'*	EUROFINS GENOMICS
SLUG	*F:5'-CATCTTTGGGGCGAGTGAGT-3'* *R:5'-GGGGTCTGAAAGCTTGGACT-3'*	EUROFINS GENOMICS
MMP9	*F:5'-CACTGTCCACCCCTCAGAGC-3'* *R:5'-GCCACTTGTCGGCGATAAGG-3'*	MWG
VIMENTIN	*F:5'-GAATAAGATCCTGCTGGCCGA-3'* *R:5'-TCGTTGGTTAGCTGGTCCAC-3'*	EUROFINS GENOMICS
TWIST	*F:5'-ATTCAGACCCTCAAGCTGGC-3'* *R:5'-CCTCCATCCTCCAGACCGA-3'*	EUROFINS GENOMICS
ABCG2	*F:5'-AGTTCCATGGCACTGGCCATA-3'*	CUSTOM

	R:5'-TCAGGTAGGCAATTGTGAGG-3'	
MDR1	*F:5'-GCCTGGCAGCTGGAAGACAAATAC-3'* *R:5'-ATGGCCAAAATCACAAGGGTTAGC-3'*	CUSTOM
RET	*F:5'-TCGAGGGATGCTTACTGGGA-3'* *R:5'-TATGGTCCAGGCTCCGGTTA-3'*	EUROFINS GENOMICS

F=forward; *R*=reverse

BIBLIOGRAPHY

1. Khan YS, Farhana A. Histology, Thyroid Gland. [Updated 2022 Dec 5]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan- Available from: https://www.ncbi.nlm.nih.gov/books/NBK551659/;
2.
3. InformedHealth.org [Internet]. Cologne, Germany: Institute for Quality and Efficiency in Health Care (IQWiG); 2006-. How does the thyroid gland work? 2010 Nov 17 [Updated 2018 Apr 19]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK279388/;
4. Anupama Sapkota. Thyroid Gland- Definition, Structure, Hormones, Functions, Disorders, The Biology Notes. https://thebiologynotes.com/thyroid-gland/;
5. Al-Suhaimi EA, Khan FA. (2022). Thyroid Glands: Physiology and Structure. In: Al-Suhaimi, E.A. (eds) Emerging Concepts in Endocrine Structure and Functions. Springer, Singapore. https://doi.org/10.1007/978-981-16-9016-7_5;
6. Cheng F, Xiao J, Shao C, Huang F, Wang L, Ju Y, Jia H. Burden of Thyroid Cancer From 1990 to 2019 and Projections of Incidence and Mortality Until 2039 in China: Findings From Global Burden of Disease Study. Front Endocrinol (Lausanne). 2021 Oct 6;12:738213. doi:10.3389/fendo.2021.738213. PMID: 34690931; PMCID: PMC8527095;
7. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021: 71: 209- 249. https://doi.org/10.3322/caac.21660;
8. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, Pacini F, Randolph GW, Sawka AM, Schlumberger M, Schuff KG, Sherman SI, Sosa JA, Steward DL, Tuttle RM, Wartofsky L. 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer: The American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. Thyroid. 2016 Jan;26(1):1-133. doi: 10.1089/thy.2015.0020. PMID: 26462967; PMCID: PMC4739132;
9. Cakir M, Grossman AB: Medullary Thyroid Cancer: Molecular Biology and Novel Molecular Therapies. Neuroendocrinology 2009;90:323-348. doi: 10.1159/000220827;

10. Elisei R, Tacito A, Ramone T, Ciampi R, Bottici V, Cappagli V, Viola D, Matrone A, Lorusso L, Valerio L, Giani C, Campopiano C, Prete A, Agate L, Molinaro E, Romei C. Twenty-Five Years Experience on RET Genetic Screening on Hereditary MTC: An Update on The Prevalence of Germline RET Mutations. Genes (Basel). 2019 Sep 10;10(9):698. doi: 10.3390/genes10090698. PMID: 31510104; PMCID: PMC6771015;
11. Prete A, Borges de Souza P, Censi S, Muzza M, Nucci N, Sponziello M. Update on Fundamental Mechanisms of Thyroid Cancer. Front Endocrinol (Lausanne). 2020 Mar 13;11:102. doi: 10.3389/fendo.2020.00102. PMID: 32231639; PMCID: PMC7082927;
12. Taccaliti A, Silvetti F, Palmonella G, Boscaro M. Genetic alterations in medullary thyroid cancer: diagnostic and prognostic markers. Curr Genomics. 2011 Dec;12(8):618-25. doi: 10.2174/138920211798120835. PMID: 22654561; PMCID: PMC3271314;
13. Romei C, Elisei R. RET/PTC Translocations and Clinico-Pathological Features in Human Papillary Thyroid Carcinoma. Front Endocrinol (Lausanne). 2012 Apr 11;3:54. doi: 10.3389/fendo.2012.00054. PMID: 22654872; PMCID: PMC3356050;
14. Mulè C, Ciampi R, Ramone T, Prete A, Matrone A, Cappagli V, Torregrossa L, Basolo F, Elisei R, Romei C. Higher *RET* Gene Expression Levels Do Not Represent an Alternative *RET* Activation Mechanism in Medullary Thyroid Carcinoma. Biomolecules. 2021 Oct 19;11(10):1542. doi: 10.3390/biom11101542. PMID: 34680178; PMCID: PMC8533768;
15. Bhattarai C, Poudel PP, Ghosh A, Kalthur SG. The *RET* gene encodes RET protein, which triggers intracellular signaling pathways for enteric neurogenesis, and *RET* mutation results in Hirschsprung's disease. AIMS Neurosci. 2022 Mar 16;9(1):128-149. doi: 10.3934/Neuroscience.2022008. PMID: 35434281; PMCID: PMC8941195;
16. Prazeres H, Torre J, et all. How to Treat a Signal? Current Basis for RET-Genotype-Oriented Choice of Kinase Inhibitors for the Treatment of Medullary Thyroid Cancer. Journal of Thyroid Research Vol. 2011, Article ID 678357, 10 pages doi:10.4061/2011/678357;
17. Melillo RM, Cirafici AM, De Falco V, Bellantoni M, Chiappetta G, Fusco A, Carlomagno F, Picascia A, Tramontano D, Tallini G, Santoro M. The oncogenic activity of RET point mutants for follicular thyroid cells may account for the occurrence of papillary thyroid carcinoma in patients affected by familial medullary thyroid carcinoma. Am J Pathol. 2004 Aug;165(2):511-21. doi: 10.1016/S0002-9440(10)63316-0. PMID: 15277225; PMCID: PMC1618571;

18. Kouvaraki MA, Shapiro SE, Perrier ND, Cote GJ, Gagel RF, Hoff AO, Sherman SI, Lee JE, Evans DB. RET proto-oncogene: a review and update of genotype-phenotype correlations in hereditary medullary thyroid cancer and associated endocrine tumors. Thyroid. 2005 Jun;15(6):531-44. doi: 10.1089/thy.2005.15.531. PMID: 16029119;
19. Plaza-Menacho I, Barnouin K, Goodman K, Martínez-Torres RJ, Borg A, Murray-Rust J, Mouilleron S, Knowles P, McDonald NQ. Oncogenic RET kinase domain mutations perturb the autophosphorylation trajectory by enhancing substrate presentation in trans. Mol Cell. 2014 Mar 6;53(5):738-51. doi: 10.1016/j.molcel.2014.01.015. Epub 2014 Feb 20. PMID: 24560924; PMCID: PMC3988870;
20. Fallahi P, Ferrari SM, Elia G, Ragusa F, Paparo SR, Ruffilli I, Patrizio A, Materazzi G, Antonelli A. Evaluating vandetanib in the treatment of medullary thyroid cancer: patient-reported outcomes. Cancer Manag Res. 2019 Aug 21;11:7893-7907. doi: 10.2147/CMAR.S127848. PMID: 31686907; PMCID: PMC6708888;
21. Thein KZ, Velcheti V, Mooers BHM, et all. Precision therapy for RET-altered cancers with RET inhibitors. Trends in Cancer, Dec.2021, Vol. 7, No. 12 https://doi.org/10.1016/j.trecan.2021.07.003;
22. Gild ML, Tsang VHM, Clifton-Bligh RJ. et all. Multikinase inhibitors in thyroid cancer: timing of targeted therapy. Nat Rev Endocrinol **17**, 225–234 (2021). https://doi.org/10.1038/s41574-020-00465-y;
23. Brglez Mojzer E, Knez Hrnčič M, Škerget M, Knez Ž, Bren U. Polyphenols: Extraction Methods, Antioxidative Action, Bioavailability and Anticarcinogenic Effects. Molecules. 2016 Jul 11;21(7):901. doi: 10.3390/molecules21070901. PMID: 27409600; PMCID: PMC6273793;
24. Panche AN, Diwan AD, Chandra SR. Flavonoids: an overview. J Nutr Sci. 2016 Dec 29;5:e47. doi: 10.1017/jns.2016.41. PMID: 28620474; PMCID: PMC5465813;
25. Arranz S, Chiva-Blanch G, Valderas-Martínez P, Medina-Remón A, Lamuela-Raventós RM, Estruch R. Wine, beer, alcohol and polyphenols on cardiovascular disease and cancer. Nutrients. 2012 Jul;4(7):759-781. doi: 10.3390/nu4070759. Epub 2012 Jul 10. PMID: 22852062; PMCID: PMC3407993;
26. Maleki Dana P, Sadoughi F, Asemi Z. et all. The role of polyphenols in overcoming cancer drug resistance: a comprehensive review. Cell Mol Biol Lett 27, 1 (2022). https://doi.org/10.1186/s11658-021-00301-9;
27. Das J, Ramani R, Suraju MO. Polyphenol compounds and PKC signaling. Biochim Biophys Acta. 2016 Oct;1860(10):2107-21. doi:

10.1016/j.bbagen.2016.06.022. Epub 2016 Jun 29. PMID: 27369735; PMCID: PMC4961512;
28. Costa M, Sezgin-Bayindir Z, Losada-Barreiro S, Paiva-Martins F, Saso L, Bravo-Díaz C. Polyphenols as Antioxidants for Extending Food Shelf-Life and in the Prevention of Health Diseases: Encapsulation and Interfacial Phenomena. Biomedicines. 2021 Dec 14;9(12):1909. doi: 10.3390/biomedicines9121909. PMID: 34944722; PMCID: PMC8698762;
29. D'Archivio M, Santangelo C, Scazzocchio B, Varì R, Filesi C, Masella R, Giovannini C. Modulatory effects of polyphenols on apoptosis induction: relevance for cancer prevention. Int J Mol Sci. 2008 Mar;9(3):213-228. doi: 10.3390/ijms9030213. Epub 2008 Feb 28. PMID: 19325744; PMCID: PMC2635670;
30. Xie X, Yin J, Jia Q, Wang J, Zou C, Brewer KJ, Colombo C, Wang Y, Huang G, Shen J. Quercetin induces apoptosis in the methotrexate-resistant osteosarcoma cell line U2-OS/MTX300 via mitochondrial dysfunction and dephosphorylation of Akt. Oncol Rep. 2011 Sep;26(3):687-93. doi: 10.3892/or.2011.1328. Epub 2011 May 26. PMID: 21617882;
31. Rajendran P, Abdelsalam SA, Renu K, Veeraraghavan V, Ben Ammar R, Ahmed EA. Polyphenols as Potent Epigenetics Agents for Cancer. Int J Mol Sci. 2022 Oct 3;23(19):11712. doi: 10.3390/ijms231911712. PMID: 36233012; PMCID: PMC9570183;
32. Hassan FU, Rehman MS, Khan MS, Ali MA, Javed A, Nawaz A, Yang C. Curcumin as an Alternative Epigenetic Modulator: Mechanism of Action and Potential Effects. Front Genet. 2019 Jun 4;10:514. doi: 10.3389/fgene.2019.00514. PMID: 31214247; PMCID: PMC6557992;
33. Jiang A, Wang X, Shan X, Li Y, Wang P, Jiang P, Feng Q. Curcumin Reactivates Silenced Tumor Suppressor Gene RARβ by Reducing DNA Methylation. Phytother Res. 2015 Aug;29(8):1237-45. doi: 10.1002/ptr.5373. Epub 2015 May 15. PMID: 25981383;
34. Shukla S, Ohnuma S, Ambudkar SV. Improving cancer chemotherapy with modulators of ABC drug transporters. Curr Drug Targets. 2011 May;12(5):621-30. doi: 10.2174/138945011795378540. PMID: 21039338; PMCID: PMC3401946;
35. Qian F, Wei D, et all., Modulation of P-glycoprotein function and reversal of multidrug resistance by (–)-epigallocatechin gallate in human cancer cells, Biomedicine & Pharmacotherapy, Volume 59, Issue 3, 2005,Pages 64-69, https://doi.org/10.1016/j.biopha.2005.01.002;
36. Kweon SH, Song JH, Kim TS. Resveratrol-mediated reversal of doxorubicin resistance in acute myeloid leukemia cells via downregulation

of MRP1 expression. Biochem Biophys Res Commun. 2010 Apr 23;395(1):104-10. doi: 10.1016/j.bbrc.2010.03.147. Epub 2010 Mar 27. PMID: 20350534;
37. Vinod BS, Maliekal TT, et all. Phytochemicals As Chemosensitizers: From Molecular Mechanism to Clinical Significance. VOL.18, NO.11 Comprehensive Invited Reviews normal Published Online: 25 Feb 2013 https://doi.org/10.1089/ars.2012.4573;
38. Cho YA, Lee W, Choi JS. Effects of curcumin on the pharmacokinetics of tamoxifen and its active metabolite, 4-hydroxytamoxifen, in rats: possible role of CYP3A4 and P-glycoprotein inhibition by curcumin. Pharmazie. 2012 Feb;67(2):124-30. PMID: 22512082;
39. Lu JJ, Pan W, Hu YJ, Wang YT. Multi-target drugs: the trend of drug research and development. PLoS One. 2012;7(6):e40262. doi: 10.1371/journal.pone.0040262. Epub 2012 Jun 29. PMID: 22768266; PMCID: PMC3386979;
40. Wang X, Zhang H, Chen X. Drug resistance and combating drug resistance in cancer. Cancer Drug Resist. 2019;2(2):141-160. doi: 10.20517/cdr.2019.10. Epub 2019 Jun 19. PMID: 34322663; PMCID: PMC8315569;
41. Renaud J and Martinoli MG. Considerations for the Use of Polyphenols as Therapies in Neurodegenerative Diseases. Int J Mol Sci. 2019 Apr; 20(8): 1883;
42. Brglez Mojzer E, Knez Hrnčič M, Škerget M, Knez Ž, Bren U. Polyphenols: Extraction Methods, Antioxidative Action, Bioavailability and Anticarcinogenic Effects. Molecules. 2016 Jul 11;21(7):901. doi: 10.3390/molecules21070901. PMID: 27409600; PMCID: PMC6273793;
43. Renaud J, Martinoli MG. Considerations for the Use of Polyphenols as Therapies in Neurodegenerative Diseases. Int J Mol Sci. 2019 Apr 16;20(8):1883. doi: 10.3390/ijms20081883. PMID: 30995776; PMCID: PMC6514961;
44. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res. 2010 Jan 15;70(2):440-6. doi: 10.1158/0008-5472.CAN-09-1947. Epub 2010 Jan 12. PMID: 20068163;
45. Verrienti A, Tallini G, Colato C, Boichard A, Checquolo S, Pecce V, Sponziello M, Rosignolo F, de Biase D, Rhoden K, Casadei GP, Russo D, Visani M, Acquaviva G, Ferdeghini M, Filetti S, & Durante C. (2016). RET mutation and increased angiogenesis in medullary thyroid carcinomas, Endocrine-Related Cancer, 23(8), 665-676. Retrieved Apr 23, 2023, from https://doi.org/10.1530/ERC-16-0132;

46. Measuring the DNA Content of Cells in Apoptosis and at Different Cell-Cycle Stages by Propidium Iodide Staining and Flow Cytometry. Cold Spring Harb Protoc; 2016; doi:10.1101/pdb.prot087247;
47. Roth V. 2006 Doubling Time Computing, Available from http://www.doubling-time. com/compute.php;
48. Livak KJ, Schmittgen TD et all. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Method. Methods. 2001 Dec;25(4):402-8;
49. Mazzaferri EL, Jhiang SM. Long-term impact of initial surgical and medical therapy on papillary and follicular thyroid cancer. Am J Med. 1994 Nov;97(5):418-28. doi: 10.1016/0002-9343(94)90321-2. Erratum in: Am J Med 1995 Feb;98(2):215. PMID: 7977430;
50. Emran TB, Shahriar A, Mahmud AR, Rahman T, Abir MH, Siddiquee MF, Ahmed H, Rahman N, Nainu F, Wahyudin E, Mitra S, Dhama K, Habiballah MM, Haque S, Islam A and Hassan MM (2022) Multidrug Resistance in Cancer: Understanding Molecular Mechanisms, Immunoprevention and Therapeutic Approaches. Front. Oncol. 12:891652. doi: 10.3389/fonc.2022.891652;
51. Alqahtani T, Alswied A, Sun, D. Selective Antitumor Activity of Datelliptium toward Medullary Thyroid Carcinoma by Downregulating RET Transcriptional Activity. Cancers 2021, 13, 3288. https://doi.org/10.3390/ cancers13133288;
52. Xiao H, Zheng Y, Ma L, Tian L and Sun Q (2021) Clinically-Relevant ABC Transporter for Anti-Cancer Drug Resistance. Front. Pharmacol. 12:648407. doi: 10.3389/fphar.2021.648407;
53. American Journal of Roentgenology. 2013;201: W867-W876. 10.2214/AJR.12.1037, https://www.ajronline.org/doi/full/10.2214/AJR.12.10370;
54. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res. 2010 Jan 15;70(2):440-6. doi: 10.1158/0008-5472.CAN-09-1947. Epub 2010 Jan 12. PMID: 20068163;
55. Center for drug evaluation and research application number: 022405Orig1s000 Clinical pharmacology and biofharmaceutics reviw(s). NDA 22-405 Submission Date: 7 July 2010;
56. Hussain, S., & Alhadidi, Q. M. (2022). Polyphenols as Tyrosine Kinase Inhibitors for the Treatment of Metastatic Cancers: Current and Future Perspective. Indonesian Journal of Pharmacy, 33(3), 366-380. https://doi.org/10.22146/ijp.3844;

57. Danışman Kalındemirtaş F, Birman H, Candöken E, Bilgiş Gazioğlu S, Melikoğlu G, Kuruca S. Cytotoxic Effects of Some Flavonoids and Imatinib on the K562 Chronic Myeloid Leukemia Cell Line: Data Analysis Using the Combination Index Method. Balkan Med J. 2019 Feb 28;36(2):96-105. doi: 10.4274/balkanmedj.galenos.2018.2017.1244. Epub 2018 Nov 5. PMID: 30396879; PMCID: PMC6409953.