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Novel Combinatorial Therapies To Target Colorectal Cancer Stem Cells

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Introduction

Cancer progression is a multifaceted process characterized by the acquisition of genetic and phenotypic changes that enable tumor growth, invasion, and metastasis. Central to this progression is the ability of cancer cells to adapt and survive in hostile environments, a process significantly influenced by the emergence of chemoresistance and the role of cancer stem cells (CSCs). Historically, the stochastic model proposed that all cancer cells within a tumor possess the potential to initiate tumors and drive progression, assuming a degree of biological equivalence among the cells. However, this model has been increasingly challenged by the cancer stem cell hypothesis, which posits that only a distinct subpopulation of cells—CSCs—are endowed with the capacity for self-renewal, differentiation, and sustained tumor initiation.

Stochastic and CSC models differ fundamentally in their approach to predicting tumor growth. The Stochastic model views tumors as biologically homogeneous, where every cell has an equal chance of acquiring mutations that drive tumor growth. It emphasizes the role of random genetic mutations and environmental factors in tumor development and progression. It incorporates randomness to account for the variability in cellular processes such as reproduction, growth, and death. This model is useful for understanding the dynamics of tumor-immune interactions and treatment responses, as it considers the probabilistic nature of cellular events and environmental influences on tumor growth. In contrast, the CSC model posits that only a specific subset of cells, known as cancer stem cells, have the ability to self-renew and sustain tumor growth. This model suggests a hierarchical organization within tumors, where CSCs are at the apex, driving tumorigenesis and metastasis. These CSCs are thought to arise from mutations in normal stem cells or through de-differentiation of differentiated cancer cells. The model highlights the role of CSCs in therapy resistance and recurrence. Overall, while the stochastic model focuses on genetic variability and randomness, the CSC model highlights cellular hierarchy and the pivotal role of stem-like cells in cancer progression.

Combining CSC and stochastic models for personalized cancer treatment offers several potential benefits:

- Enhanced Understanding of Tumor Heterogeneity: The combined model allows for a more comprehensive understanding of tumor heterogeneity by simulating the dynamic interactions between CSCs and non-stem cancer cells, including stochastic transitions that contribute to tumor diversity.
- Improved Therapy Design: By capturing both the hierarchical nature of CSCs and the random genetic mutations emphasized in stochastic models, this approach helps in designing therapies that target both CSCs and the broader tumor cell population, potentially improving treatment efficacy and reducing recurrence.
- Predictive Power for Treatment Outcomes: The integration of these models can simulate various therapeutic scenarios, predicting how different treatments might affect CSC dynamics and tumor growth, thus aiding in the personalization of therapy protocols.

Emerging evidence suggests that CSCs play a pivotal role in driving metastatic disease, the primary cause of cancer-related mortality. Unlike the majority of tumor cells, which may display limited replicative and migratory potential, CSCs are highly plastic and capable of adapting to environmental pressures such as chemotherapy, radiation, and immune responses. This adaptability often results in the selective survival of CSCs following treatment, leading to recurrence and resistance. CSCs exhibit unique characteristics, including the expression of stem cell-associated markers (e.g., CD44, CD133, ALDH1) and activation of key signaling pathways such as Wnt/β-catenin, Notch, and Hedgehog.

These pathways not only sustain the self-renewal and differentiation capabilities of CSCs but also enhance their migratory and invasive properties, facilitating metastatic colonization.

CSCs are also key drivers of therapy resistance due to their unique ability to evade conventional treatments through quiescence, enhanced DNA repair, and the expression of drug efflux pumps. Targeting CSCs has therefore become a focal point in overcoming resistance and preventing relapse. Novel therapeutic strategies include inhibitors of CSC-specific signaling pathways, such as Wnt/β-catenin, Notch, and Hedgehog, which are critical for maintaining their self-renewal and survival. Additionally, drugs targeting metabolic vulnerabilities, like mitochondrial inhibitors and glycolysis blockers, are being explored to disrupt the energy supply of CSCs. Immunotherapies, such as CAR-T cells and immune checkpoint inhibitors tailored to CSC-specific antigens, are also gaining traction. Furthermore, epigenetic modulators and agents targeting the CSC microenvironment—like hypoxia-inducing factor (HIF) inhibitors—offer promising avenues to sensitize CSCs to therapy. These innovative approaches hold potential to eradicate CSCs, reduce therapy resistance, and improve patient outcomes. The cancer stem cell concept has redefined the understanding of intratumoral heterogeneity and its implications for therapeutic resistance. Unlike the stochastic model, which fails to account for the selective and hierarchical nature of tumor initiation, the CSC hypothesis aligns with clinical observations of tumor relapse and metastatic dormancy. Recent studies highlight the dynamic interplay between CSCs and their microenvironment, including interactions with stromal cells, immune evasion mechanisms, and metabolic reprogramming, which collectively sustain the metastatic niche. Importantly, CSCs exhibit resistance to conventional therapies by virtue of quiescence, efficient DNA repair mechanisms, and expression of drug efflux transporters.

In this context, natural compounds have emerged as a promising avenue in anticancer therapies due to their diverse bioactive properties, minimal toxicity, and ability to target multiple pathways simultaneously. Derived from plants, microbes, and marine organisms, these compounds exhibit a wide range of anticancer activities, including induction of apoptosis, inhibition of angiogenesis, and suppression of metastasis. For example, curcumin, resveratrol, and paclitaxel have shown potent effects against cancer cells by modulating key signaling pathways like NF-κB, PI3K/Akt, and MAPK. Moreover, many natural compounds have demonstrated efficacy in targeting cancer stem cells (CSCs), overcoming drug resistance, and sensitizing tumors to conventional therapies. Their use in combination with chemotherapy or as standalone agents is being actively explored in clinical trials, underscoring their potential to enhance treatment efficacy and improve patient outcomes.

Another important strategy could be to target specific prognostic biomarkers, such as CD44v6. Indeed, targeting CD44v6, a splice variant of the CD44 adhesion molecule, has emerged as a promising therapeutic strategy in cancer due to its critical role in tumor progression, metastasis, and CSC function. CD44v6 is overexpressed in various malignancies, including colorectal, pancreatic, and breast cancers, where it contributes to enhanced cell migration, invasion, and resistance to therapies by activating key signaling pathways such as Ras-MAPK and PI3K-Akt. Therapeutic approaches specifically targeting CD44v6 include monoclonal antibodies, peptide-based inhibitors, and antibody-drug conjugates designed to selectively bind and neutralize this isoform. Additionally, CD44v6-specific therapies have shown potential in delivering cytotoxic agents or imaging tracers directly to tumor sites, minimizing off-target effects. These targeted strategies hold promise for disrupting CSC-mediated metastasis and overcoming therapeutic resistance, paving the way for improved outcomes in aggressive cancers.

In summary, the cancer stem cell paradigm represents a shift in understanding tumor progression and therapy resistance, emphasizing the need for targeted approaches to eradicate CSCs. As research

continues to elucidate the mechanisms underlying CSC-driven metastasis, novel strategies aimed at disrupting CSC-specific pathways or re-sensitizing them to existing therapies hold promise for improving outcomes in metastatic cancer.

Chapter 1

Destroying the shield of cancer stem cells: natural compounds as promising players in cancer therapy

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Review

Destroying the Shield of Cancer Stem Cells: Natural Compounds as Promising Players in Cancer Therapy

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Abstract: In a scenario where eco-sustainability and a reduction in chemotherapeutic drug waste are certainly a prerogative to safeguard the biosphere, the use of natural products (NPs) represents an alternative therapeutic approach to counteract cancer diseases. The presence of a heterogeneous cancer stem cell (CSC) population within a tumor bulk is related to disease recurrence and therapy resistance. For this reason, CSC targeting presents a promising strategy for hampering cancer recurrence. Increasing evidence shows that NPs can inhibit crucial signaling pathways involved in the maintenance of CSC stemness and sensitize CSCs to standard chemotherapeutic treatments. Moreover, their limited toxicity and low costs for large-scale production could accelerate the use of NPs in clinical settings. In this review, we will summarize the most relevant studies regarding the effects of NPs derived from major natural sources, e.g., food, botanical, and marine species, on CSCs, elucidating their use in pre-clinical and clinical studies.

Keywords: natural products; cancer stem cells; drug resistance; alkaloids; flavonoids; polyphenols; adjuvant treatments

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1. Introduction

Despite prominent advances in the field of cancer prevention and early diagnosis, it is expected that one in five people will develop cancer during their lifespan. One of the greatest challenges in translational oncology is limiting the onset of primary or acquired drug resistance, which is boosted by cancer stem cells (CSCs). CSCs represent a pluripotent heterogeneous population within tumor bulk, with self-renewal and differentiation abilities, contributing to the failure of conventional therapies and, therefore, to disease relapse and metastasis [1]. Increasing evidence points out that different natural products (NPs) can modulate the CSCs' hallmarks and sensitize them to conventional treatment [2]. NPs show minimal side effects in comparison with chemotherapeutics, and many studies have demonstrated their emerging role as adjuvant agents in cancer treatment. In this review, we point out the potential effects of major NPs derived from different origins (dietary, botanical, and marine sources) on CSCs in pre-clinical and clinical settings.

2. Cancer Stem Cells: The Main Players in Drug Resistance

Drug resistance is doubtless the main challenge of treatment in cancer patients. It is possible to distinguish two categories of drug resistance: intrinsic resistance and acquired resistance after drug treatment [3]. Compelling evidence highlights that intratumoral

heterogeneity is one of the major hurdles involved in intrinsic drug resistance, in which the CSCs represent the main players due to their self-renewal and differentiation abilities [4,5]. The presence of CSCs has been characterized in different tumors, such as thyroid, colorectal, breast, prostate, and other solid tumors [6,7]. CSCs are identified and can be isolated by the expression of specific surface markers such as CD133 [8], CD44 [9], CD44v6 [10], Ep-CAM [11], or enzyme activity such us ALDH [12]. CSCs are also defined as tumor-initiating cells, as they can generate tumor xenografts in immunocompromised mice models [13]. Moreover, the failure of conventional therapies, based on the use of radiotherapy and chemotherapy to induce DNA damage in highly proliferative cells and to eradicate tumor mass, is strictly due to the presence of CSCs, which are characterized by multiple survival mechanisms [14]. In particular, the mechanisms through which CSCs escape chemotherapeutic treatments are different, such as i) drug export (the aberrant expression of ATP-binding cassette, ABC, drug pumps); ii) high survival (the inhibition of antiapoptotic processes, the high expression of proteins involved in DNA-damage repair, high telomerase activity); iii) reactive oxygen species (ROS) decrease (high ALDH activity, high expression of detoxification enzymes);and iv) the aberrant activation of pathways involved in stemness [15,16].

2.1. Drug Export in CSCs

It is common knowledge that the high expression of ABC proteins contributes to chemotherapy resistance and that CSCs overexpress different drug-transporter pumps, including ABCB1, ABCG2, and ABCC1 [15,17]. The Hoechst 33342 side population assay is a useful method to identify and isolate the CSC subpopulation in solid and hematopoietic tumors [18]. Yin et al. reported that $CD133^+$ $EPCAM^+$ liver CSCs express high levels of ABCG2 and ABCB1 and are highly resistant to doxorubicin treatment. The use of specific ABC inhibitors increases doxorubicin intracellular efflux, decreasing the sphere-forming capacity and viability of CSCs [19]. Other ABC transporters, including multidrug resistance protein 1 (MRP1, ABCC1), breast cancer resistance protein (BCRP), and MRP5/ABCC5, are reported as multidrug resistance transporters in solid and hematopoietic tumors [20–22]. Moreover, $CD133^+$ melanoma CSCs expressed higher levels of ABCB5 compared to $CD113^-$ cells and are resistant to the antiapoptotic activity of the natural compound caffeic acid phenethyl ester [23]. In the lung, the high expression levels of ABCB1 in CSCs mediated the resistance to PHA-665752 and crizotinib, a MET inhibitor [24]. Although the targeting of ABC transporters could be an effective strategy to target CSCs, the use of specific inhibitors causes many side effects, due to the expression of the same targets in normal cells, as well [24].

2.2. Enhanced Survival Ability in CSCs

CSCs can also circumvent the toxic effects induced by chemotherapeutic treatment activating DNA damage response (DDR) by the ATM(ataxia-telangiectasia-mutated)- and ATR (ATM- and RAD3-related)-dependent phosphorylation of targets such as Check-1, Check-2, or H2A.X (known as γH2A.X when phosphorylated) [25]. Manic and co-workers demonstrated that in colorectal CSCs the treatment with chemotherapeutic agents induces the activation of the DDR players, such as PARP1, RAD51, and/or MRE11, resulting in higher DNA damage repair machinery [26]. In breast cancer, both $BRCA1^{wt}$ and $BRCA1^{mut}$ CSCs were highly resistant to PARP inhibitors, due to the high expression of Rad51 and Sam68, and the inhibition of this critical signaling axis hampered CSC viability [27,28]. In addition, $CD133^+$ glioma CSCs displayed resistance to radiotherapy treatment by the activation of DNA-damage repair mechanisms, where Check-1 and Check-2 are the main players. The inhibition of these two effectors reverted the radioresistance in glioma-CSCs, suggesting that targeting DNA damage could be a promising therapeutic approach for brain cancer treatment [29].

The deregulation of apoptotic pathways is another mechanism underlying CSC-mediated chemoresistance. A weak expression of death receptors, such as TRAIL and FAS,

and the overexpression of inhibitor apoptosis proteins (IAPs) have been described in CSCs compared to differentiated tumor cells [6,30]. In CSCs, IAPs are often overexpressed and impair the activation of the apoptosis cascade by mediating pro-apoptotic protein degradation [31]. $CD133^+$ colorectal CSCs highly resistant to 5-fluorouracil (5-FU) treatment expressed high levels of *SURVIVIN*, and the use of a specific aptamer-*SURVIVIN* siRNA enhanced the in vitro and in vivo 5-FU efficacy [32,33]. Moreover, in nasopharyngeal CSCs, XIAPs increased the stability of SOX2, and the use of an inhibitor of the IAP family in combination with 5-FU impaired tumor growth [34]. It has been reported that an aberrant expression of BCL-2 family members in CSCs contributes to drug resistance [20,35]. BCL-2 is overexpressed in leukemia stem cells and the use of a specific inhibitor of BCL-2, venetoclax, combined with azacitidine, resulted in disease remission in acute myeloid leukemia by CSC targeting [36,37]. In gastro-esophageal cancers, the use of the small molecule AT-101, which inhibits the BCL-2 family, decreased the expression of CSC markers (YAP1/SOX9) [38] (NCT00561197).

One of the effects of radiotherapy is the induction of DNA damage through the production of ROS and water-derived radicals. In CSCs, the presence of ROS is dramatically reduced due to the increase in ROS scavengers, limiting apoptosis induction and DDR mechanism activation [39]. The CSCs can also escape from anticancer therapies by increasing aldehyde dehydrogenase (ALDH) activity, which acts by reducing intracellular ROS levels. In turn, ROS generated from radio- and chemotherapy enhance the cytosolic expression of aldehydes, such as ALDH1A and 3A1 [40]. High levels of drug-metabolizing enzymes, such as ALDH1a1 and bleomycin hydrolase (BLMH1), have been characterized in the secretome of colorectal CSCs, increasing chemoresistance [41]. Moreover, several studies have pointed out that CSCs isolated from different tumor types display high ALDH expression levels and activity, which boost their chemoresistance [12,42]. Therefore, the upregulation of ROS levels could be an efficient strategy to counteract CSC features and sensitize CSCs to treatments.

2.3. Stemness Induction in CSCs by Different Signaling Pathways

Several signaling pathways, among which are Notch, Sonic-Hedgehog (SHH), Wnt/β-catenin, PI3K/Akt/mTOR (mTORC1 and mTORC2), TGF-β, JACK/STAT, and Hippo-YAP/TAZ, are aberrantly activated or deregulated in CSCs compared to normal stem cells [43].

The Wnt signaling pathway plays a key role during embryogenesis, and in many cancers, such as breast, colorectal, thyroid, and esophageal cancers, its activation promotes CSC growth and chemoresistance [43,44]. It has been demonstrated that the Wnt pathway is crucial for the maintenance of intestinal crypt homeostasis, and the APC mutation in transgenic mice increased the presence of $LGR5^+$ stem cells at the bottom of crypts, boosting the transformation in microadenoma [45]. Vermeulen et al. demonstrated that the Wnt pathway is highly activated in $CD133^+$ colorectal CSCs and can be influenced by extrinsic factors secreted by TME cells [46]. In hepatocellular carcinoma, the activation of the Wnt signaling pathway, induced by protein tyrosine kinase-2 (PTK2), boosted CSC tumorigenic potential and contributed to sorafenib resistance [47]. In endometrial CSCs, Lu and co-workers showed that SPARC-related modular calcium binding 2 (SMOC-2) interacts with Fzd6 and LRP6 (LDL-receptor-related protein 6) receptors and activates the Wnt/β-catenin pathway, increasing cisplatin and placlitaxel resistance [48].

The SHH signaling pathway is involved in normal embryogenesis development and plays a key role in the promotion of tumor growth and in drug resistance, upregulating the genes involved in CSC maintenance, such as *CD44*, *CCND2*, *c-MYC*, *NANOG*, *OCT4*, and *ALDH1* [49,50]. The SHH signaling pathway is involved in chemoresistance mechanisms by the modulation of the ABCG2 transporter and ALDH activity [51,52].

The dual TGF-β role in tumor progression has been extensively studied [53]. In fact, TGF-β is a key regulator of stemness, promoting EMT and radio-/chemoresistance [54,55]. Moreover, it has been shown that the cooperation of TGF-β with other signaling pathways

increases CSC features. TGF-β and tumor necrosis factor alpha (TNF-α) induced a mesenchymal phenotype in breast CSCs by decreasing *CLDN3-4-7* gene expression and, in turn, increasing in vivo tumorigenesis and resistance to oxaliplatin, etoposide, and paclitaxel [56]. In leukemia stem cells, TGF-β regulated the activation of AKT and induced FOXO3a nuclear localization, boosting sphere-forming ability and tumor growth [57].

In addition to the mechanisms described above, other intrinsic and extrinsic factors contribute to drug resistance in CSCs. Increasing evidence sheds new light on the role of epigenetic alterations in increasing intratumoral heterogeneity and in the failure of standard therapies [58]. Several molecular mechanisms, such as DNA methylation, chromatin remodeling, regulation by non-coding RNAs, and the modification of histone proteins, contribute to the aberrant expression of ABC transporters in solid and hematological tumors [1,59]. Furthermore, numerous studies point out that the crosstalk between CSCs and the tumor microenvironment (TME) influences the plasticity of CSCs, promoting drug resistance [6,60].

To overcome this challenge in cancer treatment, many researchers have focused on the development of therapeutic approaches targeting CSCs and the different mechanisms involved in drug resistance. In this regard, NPs could be considered eligible candidates.

3. Natural Products as Adjuvant against Cancer Stem Cells

In recent years, due to rising drug costs and the need to protect the environment and give ecological credentials to chemotherapy compounds, the use of NPs is constantly growing and developing. In particular, NPs contain active compounds, which might affect multiple signaling pathways involved in self-renewal and the maintenance of CSCs, with limited side effects [61]. The use of these compounds in medicine has a historic background; in fact, the use of NPs has been reported since the time of the Egyptians for disease treatment. NPs can be extracted from several parts of plants, such as the root, stem, fruit, and leaf; dietary agents; and marine organisms [62,63].

The pharmaceutical industry shows great interest in NPs due to their unique properties, such as high diversity and steric complexity, lighter atoms, and low hydrophobicity [64]. These structural features can be used to synthesize commercial drugs useful for both cancer prevention and treatment [65]. Recent in vitro and in vivo studies have demonstrated that NPs counteract cancer progression by interfering with the self-renewal capacity of CSCs, the induction of apoptosis, the inhibition of cancer-cell spreading, and the arrest of the cell cycle [61]. Moreover, several natural compounds, such as alkaloids, terpenoids, polyphenols, and flavonoids are efficient modulators of ABC transporters and sensitize CSCs to conventional chemotherapeutic treatment [66].

To date, about 500 clinical trials are registered on the clinical trial.gov website, reporting the effects of NPs and NP-derived drugs for the treatment of different cancers (www.clinicaltrial.gov, accessed on 20 September 2022). Here, we report a large overview of the properties of NPs that sensitize CSCs to conventional chemotherapeutic treatments.

3.1. NPs Derived from Dietary Sources

A strong correlation has been demonstrated between tumor incidence and a correctly healthy lifestyle. Many NPs derive from food and belong to the polyphenols category. Polyphenols are characterized by the presence of aromatic benzene rings bonded to hydroxyl groups. These compounds are classified into stilbenes, lignans, tannins, flavonoids, and phenolic acids. Polyphenols show a role in the regulation of angiogenesis, inflammation, and the apoptosis of CSCs in in vitro settings [67]. Moreover, they can improve immune response by modulating T lymphocytes [68]. In this regard, recent studies have also highlighted that natural polyphenols can be used as adjuvants in association with conventional therapy to limit the CSCs' drug-resistance phenomenon [69,70].

It has been demonstrated that curcumin, a polyphenol extracted from *Curcuma longa*, has anticancer effects against different types of tumors [71]. One of the prominent anticancer activities of curcumin is the blocking of NF-kB pathways through the inhibition

of IKK activity [72]. Of note, liver cancer cells displayed different phenotypes after curcumin treatment, which can be divided into sensitivity and resistance. In sensitive cells, curcumin reduced cell viability via the reduction in CSC features, such as SP population, sphere-forming capacity, and tumorigenic potential. Conversely, curcumin treatment boosts stem-like properties in resistant cells. To identify the signaling pathways modulated by curcumin in sensitive and resistant cells, the authors performed a transcriptomic analysis which points out a downregulation of *HDACs* in sensitive cells. Curcumin, in combination with HDAC inhibitors, affected the sphere-forming ability and reduced the SP fraction in resistant cells [73]. In addition to the regulation of NF-kB pathways, curcumin modulates another key tumorigenic signal. Wu et al. reported that curcumin can inhibit the JAK2/STAT3 pathway in lung CSCs, reducing in vitro tumorsphere formation capacity and impairing tumor growth in a pre-clinical mouse model [74]. Moreover, this NP reduced the proliferation of and in turn promoted apoptosis in lung CSCs, causing a reduction in the main stemness markers through the downregulation of the Wnt/β-catenin and SHH pathways [75]. Curcumin, alone or in combination with piperine, inhibited the formation of tumorspheres in breast cancer cells, interfering with the stem-cell signaling pathways involved in carcinogenesis [76]. Moreover, curcumin could prevent the cell proliferation of $LGR5^+$ colorectal cells by triggering autophagy and blocking via the TFAP2-mediated ECM pathway [77]. In recent years, several studies have shown that natural polyphenols may be used as adjuvant therapy in association with traditional treatment to reduce CSC drug resistance [69,70]. Curcumin is associated with low doses of cisplatin-induced apoptosis and reduced the migration in the $CD166^+/EpCAM^+$ CSC subpopulation in lung cancer cells by enhancing the sensitivity of the cells to chemotherapy [78]. In thyroid cancer, the combinatorial treatment with curcumin and cisplatin impaired sphere formation and the expression of stemness markers in thyrospheres via the downregulation of the JAK/STAT3 pathway [79]. Although different in vitro and in vivo studies have highlighted the role of curcumin in sensitizing CSCs to therapy, its use in clinical settings is limited by insolubility in water and fast metabolism [80].

Resveratrol is a natural product present in several types of food, such as the skin of grapes and berries, with multiple antitumoral effects, such as the inhibition of angiogenesis and detoxification enzymes and the induction of apoptosis [81,82]. In this regard, resveratrol can be considered a promising chemopreventive cancer agent. Jang et al. reported in a skin cancer mice model that the topical administration of resveratrol prevents tumor growth [83]. In osteosarcoma, resveratrol reduced cytokine synthesis (IL-6, TNF-α, IFN-γ, and oncostatin M) and inhibited STAT3 signaling to diminish the expression of CD133CSC markers [84]. According to Ferraresi et al., resveratrol could be used as a therapeutic strategy for the treatment of ovarian cancer, reducing cell migration and viability. Specifically, resveratrol counteracted the effect mediated by lysophosphatidic acid, inhibiting SHH signaling with the reduction in *BMI1*, a polycomb ring finger transcriptional factor involved in the activation of Hedgehog and restoring the autophagy pathway [85]. In pancreatic cancer, resveratrol impaired the stem-like features and the tumorigenic and invasive capacity of cancer cells [86]. In combination with 5-FU, resveratrol decreased the survival of $CD133^+$ colorectal CSCs [87]. Another antitumoral resveratrol mechanism is the induction of oxidative stress. In breast cancer CSCs, resveratrol impaired mammosphere formation and xenograft tumor growth by inducing autophagy, with an increase in LC3-II, Beclin1, and Atg 7 expression levels, and reducing the Wnt pathway [88]. Moreover, this NP enhances the generation of ROS by overloading the mitochondrial electron transport chain, which ultimately influences cell apoptosis/necrosis and enhances cell death in colorectal cancer [89,90]. For these reasons, this compound can be effective in the inhibition of viability, tumorigenic potential, and self-renewal ability of CSCs. Of note, resveratrol modulates the crosstalk between TME and CSCs. In a multicellular TME system, resveratrol affected the interaction between colorectal CSCs and stromal cells and reduced the expression of stemness markers and sphere-forming capacity by blocking p65 NF-kB nuclear translocation [90]. The same mechanism of action has been reported in breast

cancer. Resveratrol decreased the percentage of $CD44^+/CD24^-$ subpopulations and the expression levels of SOX2 and BMI-1 in BCSCs treated with the conditioned medium of cancer-associated fibroblasts [91].

Epigallocatechin-3-gallate (EGCG), a type of catechin found in green tea, has a chemopreventive activity against different types of cancers in vitro, in vivo, and in clinical settings [92,93]. Treatment with EGCG impaired the in vivo growth of prostate, lung, and gastrointestinal cancer cells [94]. In particular, EGCG regulated the expression of CSC markers and, thus, CSC features [95,96]. Luo et al. hypothesized the use of EGCG in colon cancer prevention and treatment as dietary supplements or adjuvant therapy, due to EGCG's anti-proliferation and anti-migration properties [97]. EGCG treatment reduced the invasive capacity and induced apoptosis through the downregulation of the STAT3 pathway and the modulation of proteins involved in EMT and apoptosis, impaired Wnt pathway activation, and increased the sensitivity to 5-FU treatment in colorectal CSCs [97–99]. In lung cancer, EGCG targeted $CD133^+$ cells, decreasing the self-renewal and tumorigenic potential of CSCs through the regulation of the circadian rhythm protein CLOCK [100]. Moreover, EGCG downregulated the Wnt pathway and reduced the proliferation and stemness marker expression in lung CSCs [101].

Flavonoids are polyphenolic compounds found in nuts, teas, fruit, and vegetables with antioxidant, anti-inflammatory, and anticancer properties [102]. The classification of flavonoids depends on their level of oxidation and includes flavanones, flavones, flavanols, and anthocyanins [103]. Several studies hypothesize that diets containing a high number of flavonoids could have cancer chemopreventive effects, targeting CSCs [104].

Citrus fruits including *Citrus depressa* (shiikuwasa), and *Citrus sinensis* (oranges) contain nobiletin, a healthy dietary polymethoxylated flavone [105] with a variety of biological actions, including anti-inflammatory, anti-tumor, and neuroprotective effects [106,107]. Nobiletin enhanced the internalization of chemotherapeutic or other natural compounds viathe inhibition of ABC transporters [108,109]. In non-small cell lung cancer, treatment with nobiletin inhibited the Wnt pathway and negatively correlated with EMT and stemness, reducing CD133 and ALDH1 stem markers [110]. Our group recently demonstrated that nobiletin and xanthohumol—a prenylated flavonoid contained in hop cones—extracts reduced the viability of colorectal CSCs and synergized with FOX (5-FU plus oxaliplatin) in inducing apoptosis and reducing stemness features, such as CD44v6 expression and Wnt pathway activation [111].

Apigenin is a bioavailable flavonoid belonging to the flavone class and is present in vegetables, fruits, and drinks. Apigenin exhibits anti-inflammatory activities, antioxidant effects, and anticancer properties [112,113]. Erdogan et al. demonstrated that adjuvant therapy with apigenin enhanced the sensibility of prostate CSCs to cisplatin treatment. This combinatorial therapy increased the cisplatin-induced apoptosis via the downregulation of *BCL-2*, *SHARPIN*, and *SURVIVIN* mRNAs, and enhanced the expression levels of caspase-8, Apf-1, and P53 [114]. In breast cancer, apigenin reduced the $CD44^+/CD24^-$ subpopulation in triple-negative breast CSCs, inducing tumor shrinkage through the downregulation of YAP/TAZ activity [115]. Moreover, apigenin in combination with cisplatin reduced the tumorigenic potential of $CD133^+$ lung CSCs [116].

Quercetin is a secondary metabolite from fruit and vegetable flavonols with anti-inflammatory and antioxidant properties. Cao et al. described howquercetin-3-methyl ether impaired the sphere-forming capacity of breast CSCs by reducing the expression of stemness genes (*SOX2, NANOG*) and the Notch and PI3K/AKT pathways [117]. Moreover, quercetin inhibited the tumor growth and metastasis formation of $CD44^+/CD24^-$ CSCs and reduced the expression levels of ALDH1A and CXCR4 [118,119]. In $CD24^+/CD133^+$ pancreatic CSCs, quercetin reduced the activation of the Wnt pathway and the expression of stemness markers [120].

Naringin and naringenin are flavanones obtained from citrus fruits with anti-inflammatory, antioxidant, and antitumoral properties. These natural products display chemopreventive activity in many tumors, such as lung and colorectal cancers [121,122]. Many studies

have pointed out that naringin and naringenin hamper tumor growth and progression by inhibiting pathways involved in survival, apoptosis, ROX detoxification, autophagy, and metastasis formation [123]. By bioinformatics analyses, Hermawan et al. identified naringenin as a potential drug to target breast CSCs. Indeed, naringenin treatment decreased the sphere-forming and colony-forming capacity, migration, and expression of stemness-related genes (*CTNNB1*, *ALDH1*, *VIMENTIN*) in breast CSCs [124]. In cervical cancer spheroids, naringenin in combination with cisplatin reduced the cell viability and invasion of cancer cells [125].

Sulforaphane (SFN) is an active isothiocyanate derived from the hydrolyzation of glucoraphanin by myrosinase activity. SFN is a phytoconstituent that belongs to the *Brassicaceae* family, which includes vegetables such as cauliflower, kale, cabbage, and broccoli. Numerous manuscripts have highlighted SFN's anticancer effects in both in vitro and in vivo models in different tumors, acting as an epigenetic modulator that induces apoptosis and senescence [126,127]. Li et al. highlighted that SFN treatment decreased the percentage of $CD133^+$and $ALDH^+$ cells in lung spheroids induced after exposure to cisplatin and enhanced the in vivo antitumor effect of cisplatin [128]. In breast cancer, SFN impaired the formation of mammospheres via the reduction in $ALDH^+$ cells and the activation of the Wnt/β-catenin signaling pathway. Moreover, SFN treatment decreased tumor growth and the second engraftment of breast CSCs [129]. Castro et al. showed that SFN can modulate the cell proliferation, tumorsphere formation, cell viability, and phenotype of CSCs derived from triple-negative breast cancer and counteract the xenograft tumor growth in mice [130].

Fisetin is another flavonol found in some vegetables and fruits including onion, cucumber, apple, grape, and strawberry. Fisetin is a neuroprotective agent and acts as a chemopreventive/chemotherapeutic agent in different cancers [131]. In lung CSCs, treatment with fisetin inhibited cell growth by modulating mTOR and PI3K/AKT signaling and decreased the number of colonies in a dose-dependent manner [132]. Another study in lung cancer demonstrated that fisetin exhibits anti-invasion and anti-proliferative effects via the downregulation of CD44 and CD133 stem-like markers [133]. In Table 1, we summarize the studies previously described regarding the effect of NPs on CSCs (Table 1).

Table 1. NPs derived from dietary sources and effects on CSCs.

Natural Products (Source)	Tumor Type	Effects on CSCs	Alone or in Combination with Other Compounds	References
Curcumin (*Curcumalonga*)	Liver cancer	In vitro reduction in SP subpopulation and sphere formation.	HDAC inhibitors	[73]
	Lung cancer	In vitro reduction in tumorsphere formation and inhibition of JAK2/STAT3 pathway. In vivo impaired tumor growth.		[74]
	Lung cancer	In vitro arrest of cell proliferation, induction of apoptosis, and reduction in the main stemness markers via Wnt/β-catenin and SHH pathways downregulation.		[75]
	Breast cancer	In vitro reduction in tumorsphere formation	Piperidine	[76]
	Colorectal cancer	In vitro reduction in $LGR5^+$ cell proliferation and induction of autophagy.		[77]
	Lung cancer	In vitro induction of apoptosis, reduction in $CD166^+/EpCAM^+$ cell migration.	Cisplatin	[78]
	Thyroid cancer	In vitro impairment of sphere formation and reduction in stemness marker expression via JAK/STAT3 downregulation.	Cisplatin	[79]

Table 1. *Cont.*

Natural Products (Source)	Tumor Type	Effects on CSCs	Alone or in Combination with Other Compounds	References
Resveratrol (skin of grapes and berries)	Osteosarcoma	In vitro inhibition of STAT3 pathway and reduction in CD133 expression.		[84]
	Ovarian cancer	In vitro reduction in migration and viability, inhibition of SHH pathway, and induction of autophagy.	Lysophosphatidic acid	[85]
	Pancreatic cancer	In vitro reduction in stem-like features. In vivo reduction in tumorigenic and invasive potential.		[86]
	Colorectal cancer	In vitro reduction in $CD133^+$ cell survival.	5-FU	[87]
	Breast cancer	In vitro reduction in tumorsphere formation via autophagy induction and Wnt pathway reduction. In vivo reduction in tumor growth.		[88]
	Colorectal cancer	In vitro reduction in stemness markers and sphere formation.		[89,90]
	Breast cancer	In vitro reduction in $CD44^+/CD24^-$ subpopulations and SOX2 and BMI1 expression levels.		[91]
EGCG (green tea)	Colorectal cancer	In vitro reduction in invasive capacity and induction of apoptosis and chemosensitivity via STAT3 and Wnt pathway downregulation.		[99]
	Lung cancer	In vitro reduction in self-renewal of $CD133^+$ cells. In vivoreduction in tumorigenic potential.		[100]
	Lung cancer	In vitro downregulation of Wnt pathway and reduction in proliferation and stemness marker expression.		[101]
Nobiletin (*Citrus depressa* and *Citrus sinensis*)	Lung cancer	In vitro reduction in Wnt pathway and CD133 and ALDH1 expression levels.		[110]
	Colorectal cancer	In vitro reduction in cell viability, CD44v6 expression, Wnt activation, and induction of apoptosis.	Xanthohumol (flavonoid) and FOX	[111]
Apigenin (fruits, vegetables, and drinks)	Prostate cancer	In vitroinduction of apoptosis and inhibition of cell migration and NF-kB pathway.	Cisplatin	[114]
	Breast cancer	In vivo reduction in $CD44^+/CD24^-$ tumorigenic potential via downregulation of YAP/TAZ pathway.		[115]
	Lung cancer	In vivoreduction in $CD133^+$ tumorigenic potential.	Cisplatin	[116]
Quercetin (fruits and vegetables)	Breast cancer	In vitro reduction in tumorsphere formation and decrease in stemness gene (*SOX2*, *NANOG*) expression and Notch and PI3K/AKT pathway activation.		[117]
	Breast cancer	In vitro reduction in ALDH1A1 and CXCR4 expression levels. In vivo reduction in $CD44^+/CD24^-$ tumor growth and metastasis formation.		[118,119]
	Pancreatic cancer	In vitro reduction in Wnt pathway activation and stemness markers.		[120]
Naringine and naringenin (*Citrus fruits*)	Breast cancer	In vitro reduction in mammosphere and colony formation and migration, decrease in stem-like markers (β-catenin, ALDH1).		[124]
	Cervical cancer	In vitro reduction in cell viability and invasive capacity.	Cisplatin	[125]

Table 1. *Cont.*

Natural Products (Source)	Tumor Type	Effects on CSCs	Alone or in Combination with Other Compounds	References
Sulforaphane (*Brassicaceae*)	Lung cancer	In vitro decrease in CD133+ and ALDH+ cells.	Cisplatin	[128]
	Breast cancer	In vitro reduction in mammosphere formation and decrease in ALDH expression and Wnt/β-catenin pathway activation. In vivo decrease in tumor growth and second engraftment.		[129]
	Breast cancer	In vitro reduction in cell proliferation, tumorsphere formation, and cell viability. In vivo decrease in xenograft tumor growth.		[130]
Fisetin (vegetables and fruits)	Lung cancer	In vitro reduction in cell growth and colony formation.		[132]
	Lung cancer	In vitro reduction in proliferative and invasive capacity via the downregulation of CD44 and CD133 stem-like markers.		[133]

Abbreviations: CSCs, cancer stem cells, HDAC, histone deacetylase, SP, side population, SHH, sonic hedgehog, LGR5, Leucine-rich repeat-containing G-protein coupled receptor 5, EpCAM, epithelial cell adhesion molecule, ALDH, aldehyde dehydrogenases, CXCR4, C-X-C chemokine receptor type 4.

3.2. NPs Derived from Botanical Sources

Different drugs, derived from natural compounds, are used in clinical practice as anticancer agents. Paclitaxel isolated from *Taxus brevifolia* was one the first anticancer agents studied in ovarian and breast adenocarcinoma. Some plants can produce toxic substances such as phenol or tannin when attacked by predators [134]. In this regard, many researchers studied plants as a possible source of NPs to counteract CSCs.

Luteolin is a flavone present in about 300 plant species [135]. Luteolin impaired the expression of the stemness markers ABCG2 and CD44 and affected the ALDH1 activity and spheroid formation capacity of breast CSCs. Moreover, luteolin sensitized CSCs to taxol treatment [136]. Luteolin in combination with quercetin impaired the sphere-forming ability and the expression levels of NANOG, SOX2, and CD44 [137]. In oral CSCs, luteolin effectively reduced proliferation, ALDH activity, and CD44, inactivating the IL6/STAT3 axis [138].

Berberine (BBR) is an isoquinoline alkaloid obtained from the roots and stems of anti-inflammatory plants and can induce apoptosis by reactive oxygen generation [139,140]. In the literature, the ability of BBR as a modulator of epigenetic modification has been widely demonstrated [141,142]. Zhao et al. showed that BBR impairs the proliferation and sphere-forming capacity of colorectal CSCs by enhancing the expression levels of p27 and p21, increasing the percentage of cells in the G1/G0 phase and, in turn, reducing CD44 and CD133 markers. In line with its epigenetic modulator activity, BBR impaired the RNA m6A methylation levels. Moreover, BBR treatment affects the tumorigenic capacity of colorectal CSCs and sensitizes the cells to 5-FU and irinotecan [143]. In pancreatic cancer, BBR and gemcitabine combinatorial treatment decreased SP percentage and the expression of the stemness genes *POU5F1*, *SOX2*, and *NANOG* [144]. Moreover, BBR can counteract sphere formation, the expression of EMT stemness markers, and the activation of the *GLI1–BMI1* axis induced by chemotherapy in ovarian CSCs [145]. In neuroblastoma, BBR treatment was able to induce the expression of epithelial-like marker E-cadherin, downregulating crucial signaling pathways that regulate tumor progression, such as PI3K/Akt, TGF-β, and MAPK [146].

Vincristine is another alkaloid, derived from the Madagascar periwinkle, *Catharanthus roseus*, with anti-tumor activity. Its biological mechanism of action is based on the inhibition of microtubule aggregation and, consequently, the arrest of cell mitosis in metaphase [147]. The treatment of a neuroblastoma cell line (SH-SY5Y) with vincristine reduced cell proliferation in a dose-dependent manner by blocking the cell cycle in the G2-M phase with

increased cyclin B expression and decreased cyclin D levels. Overall, these data showed that vincristine could be a promising chemotherapeutic agent for the treatment of neuroblastoma [148]. For decades, vincristine has been used in combination with chemotherapy in different tumors, as well as acute myeloid leukemia [149], lung cancer [150] (NCT00003847), colorectal cancer [151], and breast cancer [152]. Nevertheless, its anti-proliferative effects in CSCs have been little shown. Moon et al. highlighted that vincristine influences the methylation state of the runt-related transcription factor-3 gene (*RUNX3*) in colorectal cancer. The treatment with this alkaloid induced the demethylation of *RUNX3*, leading to the recovery of *RUNX3* mRNA expression in colorectal cancer cells without affecting DNA methylation in healthy colon cells [153]. These observations suggest a potential therapeutic approach for CSC targeting.

Alkaloids gained from the bark of the *Cinchona officinalis* tree have been used for more than a century for malaria prevention and treatment. Among these alkaloids, chloroquine (CQ), derived from quinine, is a potent inhibitor of autophagy in cancer cells [154]. Cufi et al. reported that CQ treatment slightly reduced the $CD44^+/CD24^-$ stem-like subpopulation and vimentin expression in triple-negative breast cancer (TNBC) cells [155]. Moreover, CQ in combination with paclitaxel decreased ALDH-positive and $CD44^+/CD24^-$ cell subpopulations and the sphere-forming capacity of TNBC cells. The combination treatment impaired autophagy through the upregulation of LCB3-II and p62 expression levels and enhanced apoptosis and cleaved caspase-3 levels by inhibiting the JAK/STAT pathway. In in vivo settings, CQ and paclitaxel lessened in vivo tumor growth and lung metastatic foci [156]. Liang et al. showed that CQ targets breast CSCs by inducing mitochondrial depolarization and the accumulation of DNA double-strand breaks, and CQ in combination with carboplatin diminished autophagy and the expression levels of proteins involved in DNA repair machinery [157]. CQ affected the $CD133^+$ subpopulation and the tumorigenic potential of pancreatic CSCs and patient-derived xenografts (PDXs) through a mechanism not previously described. In fact, CQ inhibited the CXCL12/CXCR4 axis and SHH pathway in pancreatic- CSCs [158]. A similar effect of CQ treatment was also observed in esophageal squamous cell carcinoma CSCs [159].

Recent studies have revealed that capsaicin, derived from plants of the genus *Capsicum*, showed considerable anticancer effects [160]. Zhu et al. reported that capsaicin decreases sphere size, hampers CSC survival in a dose-dependent manner, and downregulates markers such as CD133, CD44, OCT-4, NANOG, and SOX2, typically expressed in prostate CSCs. Moreover, the authors showed that capsaicin interfered with the Wnt/β-catenin signaling pathway in prostate CSCs. Briefly, capsaicin drastically reduced GSK3 phosphorylation and avoided β-catenin's translocation into the nucleus, downregulating target genes such as *MYC* and *CCND1*. The activation of the Wnt/β-catenin pathway restored the sphere-forming ability of prostate CSCs and induced the upregulation of the above-described stemness markers [161].

A saturated derivative of capsaicin, dihydrocapsaicin (DHC), is a potent inducer of autophagy [162]. DHC-induced autophagy in a catalase-dependent manner in colon and breast cancer cell lines has been reported. Oh and co-workers showed that DHC induced breast and colorectal cancer cell arrest in G0-G1, upregulating the expression levels of autophagy-related proteins [163]. Thanks to its ability to induce autophagy, DHC could be considered a promising anticancer agent. In this regard, DHC efficiently targeted the $CD133^+$ neural cell population, inducing cell death [164] (US20090076019A1). However, due to its low bioavailability, DHC isno longer being tested as a CSC-targeting agent (Table 2).

Table 2. NPs derived from botanical sources and their effects on CSCs.

Natural Products	Tumor Type	Effects on CSCs	Alone or in Combination with Other Compounds	References
Luteolin (plants)	Breast cancer	In vitro reduction in tumorsphere formation, decrease in stemness marker (ABCG2 and CD44) expression and ALDH1 activity. In vitro reduction in cell viability.	Taxol	[136]
	Prostate cancer	In vitro reduction in tumorsphere formation and of the expression levels of stem-like markers (NANOG, SOX2, and CD44).	Quercetin	[137]
	Oral cancer	In vitro arrest of cell proliferation and migration and decrease in ALDH activity, CD44 expression levels, and IL6/STAT3 axis. In vivo reduction in tumor growth through IL-6/STAT3 signaling inactivation.		[138]
Berberine (plants)	Colorectal cancer	In vitro reduction in tumorsphere formation, cell proliferation, and CD44 and CD133 expression levels. In vivo reduction in tumor growth.	5-FU and irinotecan	[143]
	Pancreatic cancer	In vitro decrease in SP percentage and the expression of the stemness genes (*POU5F1*, *SOX2*, and *NANOG*).	Gemcitabine	[144]
	Ovarian cancer	In vitro reduction in tumorsphere formation, expression of EMT stemness marker expression, and GLI1-BMI1 axis.	Carboplatin and VP-16	[145]
	Neuroblastoma	In vitro downregulation of PI3K/Akt, TGF-β, and MAPK pathways.		[146]
Vincristine (Madagascar periwinkle)	Colorectal cancer	In vitro demethylation of RUNX3.		[153]
Cloroquine (bark of the *Cinchona officinalis* tree)	Breast cancer	In vitro reduction in $CD44^+/CD24^-$ stem-like population.		[155]
	Breast cancer	In vitro reduction in $CD44^+/CD24^-$ and $ALDH^+$ stem-like population, impairment of autophagy (increased levels of LCB3-II), and increase in apoptosis and cleaved caspase-3 levels. In vivo reduction in tumor growth and metastatic foci.	Paclitaxel Paclitaxel	[156]
	Breast cancer	In vitro induction of mitochondrial depolarization. In vitro reduction in autophagy and the expression levels of DNA repair machinery proteins.	Carboplatin	[157]
	Pancreatic cancer	In vitro inhibition of CXCL12/CXCR4 axis and SHH pathway. In vivo reduction in the tumorigenic potential of $CD133^+$ subpopulation.		[158]
	Esophageal carcinoma	In vitro reduction in the CXCR4/STAT3 axis.		[159]
Capsaicin (plants of the genus *Capsicum*)	Prostate	In vitro reduction in tumorsphere formation, cell viability, and the expression levels of stem-like markers (CD133, CD44, OCT-4, NANOG, and SOX2) and downregulation of GSK3β/β-catenin pathway.		[161]
Dihydrocapsaicin (derivate of capsaicin)	Brain	In vitro decrease in $CD133^+$ subpopulation and induction of cell death.		[164]

Abbreviations: NPs, natural products, CSCs, cancer stem cells, ABCG2, ATP binding cassette subfamily G member 2, ALDH, aldehyde dehydrogenases IL-6, interleukine- 6, SP, side population, POU5F1, POU Class 5 Homeobox 1, TGF-β, tumor growth factor- β, CXCR4, C-X-C chemokine receptor type 4, LCB3-II, microtubule associated protein 1 light chain 3 beta, CXCL12, C-X-C Motif Chemokine Ligand 12.

3.3. NPs Derived from Marine Sources

The marine microenvironment is a heterogeneous environment, characterized by unique conditions (low oxygen and sunlight, as well as high salinity and pressure) that favor the presence of micro- and macro-organisms producing particular metabolites. It has been demonstrated that these molecules with unique biochemistry structures, containing various heterocyclic rings and diverse heteroatoms, can be used to prevent and treat cancer [62]. Given the growing number of marine natural compounds (MNC) used in medicine, more researchers have focused on the structure and synthesis of analogs with anticancer properties [165].

Nortopsentin, a bis-indolyl alkaloid isolated from deep-sea sponges (*Spongosoritesruetzleri*), exhibits significant antitumor activity against P388 murine leukemia [166]. Cascioferro et al. synthesized nortopsentin analogs by introducing the substitution of a central imidazole ring with a 1,2,4-oxadiazole motif and a 7-azaindole in place of the original indole motif. Among these compounds, 1k and 1n displayed cytotoxic effects on MCF-7, Caco-2, HeLa, and HCT-116 cells. The anti-proliferative activity on MCF-7 of these compounds was associated with a pro-apoptotic activity involving chromatin condensation and membrane blebbing. Moreover, these chemicals induced a buildup of cells in the G0-G1 phase, indicating that they could influence DNA replication [167]. Similar results were obtained by Di Franco et al. using another analogous of nortopsentin, NORA234. This compound reduced, at early timepoints, the clonogenic potential and the proliferation rate of colorectal CSCs. However, NORA234'sprolonged administration drove the selection of resistant subclones, characterized by high expression levels of CD44v6 and β-catenin activity, with an increased CHK1-driven DNA damage response. Treatment with NORA234 in combination with CHK1 inhibitor enhanced apoptosis and hampered the proliferation and clonogenic capacity of colorectal CSCs together with the decrease in $CD44v6^{+}/Wnt^{high}$ subpopulations [168].

Renieramycin M (RM) is the major bis-tetrahydroisoquinolinequinone alkaloid derived from the blue sponge *Xestospongia* species. Treatment with non-toxic concentrations of RM reduced colony and spheroid formation and the expression of CD133, CD44, and ALDH1A1 stem-like markers in lung CSCs [169]. According to these findings, RM could be considered a promising anticancer compound.

Fucoxanthin and its metabolite fucoxanthinol (FxOH), carotenoids isolated from different brown algae species, exhibit beneficial cancer prevention and anticancer features [170]. Terasaki et al. demonstrated that FxOH impaired the growth, sphere-forming ability, and tumorigenic potential of$CD44^{high}/EpCAM^{high}$ colorectal CSCs by the inactivation of AKT signaling and the downregulation of PPARβ/δ and PPARγ protein expression levels [171]. Moreover, FxOH treatment reduced the expression levels of N-cadherin and vimentin EMT markers, which correlate with lessened levels of glycine and succinic acid, in colorectal CSCs [172]. Sulfated polysaccharides called fucoidans derived from brown algae have a variety of biological functions. According to Vishchuk and colleagues, sulfated (1→3)-L-fucan, obtained by *Saccharinacichorioides*, reduced the colony-formation capacity of different cancer cell lines [173].

Bryostatin-1 is a macrocyclic lactone of marine origin derived from the *Bugula neritina* invertebrate. Different pre-clinical and clinical studies demonstrated its role as an antitumor agent [174,175]. Sikorska et al. showed that among different natural compounds, bryostatin-1 promoted a differentiated state in melanoma CSCs, reducing their high proliferative rate and the $ABCB5^{+}$ subpopulation [176]. Bryostatin-1 increased the Gleevec-mediatedapoptosis of chronic myeloid leukemia SCs, reducing the fraction of G0/G1 $CD34^{+}$ cells [177] (Table 3) (Figure 1).

Table 3. NPs derived from marine sources and their effects on CSCs.

Natural Products (Source)	Tumor Type	Effects on CSCs	Alone or in Combination with Other Compounds	References
Alkaloids				
Nortopsentin (deep-sea sponges, Spongsoritesruetzleri)	Colorectal	In vitro arrest of cell proliferation (inhibition of CDK1 activity), induction of apoptosis (Caspase 3), and decrease in stem cell markers (CD44v6) and pathways (Wnt/β-catenin).	Rabusertib	[168]
Renieramycin M (blue sponge *Xestospongia* species)	Non-small-cell lung cancer	In vitro reduction in tumorsphere formation andstem-like markers (CD133, CD44, ALDH1A1).		[169]
Carotenoids				
Fucoxanthinol (brown algae)	Colorectal cancer	In vitro reduction in tumorsphere formation by the inactivation of AKT signaling and the downregulation of PPARβ/δ and PPARγ protein expression, in vitro induction of apoptosis via the reduction in cellular adhesion molecule expression.		[171]
	Colorectal cancer	In vitro decrease in proliferation pathways (JAK/STAT, PI3K/Akt, MAPK, NF-κB).		[172]
Macrolides				
Bryostatin-1 (*Bugula neritina*)	Melanoma cells	In vitro reduction in proliferation and ABCB5$^+$ subpopulation.		[176]
	Leukemia	In vitroinduction of apoptosis and reduction in CD34$^+$ cell fraction	Gleevec	[177]

Abbreviations: NPs, natural products, CSCs, cancer stem cells, CDK1, Cyclin Dependent Kinase 1, ALDH, aldehyde dehydrogenases, PPARβ/δ, peroxisome-proliferator-activated receptor β/δ, PPARγ peroxisome-proliferator-activated receptor γ.

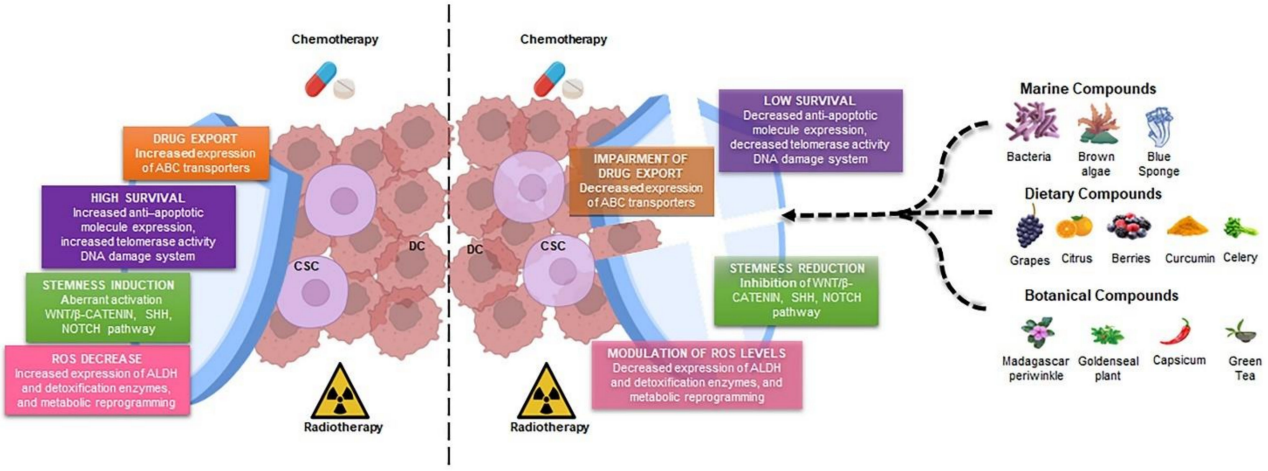

Figure 1. Natural products destroy the shield of CSCs. Radio- and chemotherapy target differentiated cancer cells, which represent most cells within the tumor mass, sparing the CSC subpopulation, characterized by the high expression of ABC transporters, anti-apoptotic molecules, and detoxification enzymes and the aberrant activation of stemness pathways and DNA repair machinery (**left part**). Natural products derived from different sources (marine, food, and botanical compounds) are able to reduce CSC features and increase their sensitivity to radio- and chemotherapy (**right part**). DCs, differentiated cells; CSC, cancer stem cell; ABC, ATP-binding cassette.

3.4. Other Natural Compounds

Other NPs such as retinoids, which do not fit into the classifications of polyphenols, flavonoids, or alkaloids, have shown promising effects for targeting CSCs [178]. Retinoids, which are classified as terpenes, induce the differentiation of CSCs, making them more sensitive to chemotherapeutic agents [179]. All-Trans Retinoic Acid (ATRA) is a biologically active compound belonging to the retinoid group and is a metabolite of vitamin A. It is essential for a variety of biological processes, such as cell division, organogenesis, differentiation, and cell death. ATRA can inhibit ALDH activity and revert MDR in CSCs. Ginestier et al. displayed that ATRA decreased mammosphere formation by regulating signaling pathways involved in CSC differentiation [180]. In glioblastoma, treatment with ATRA increased the expression levels of astrocytic (GFAP) and neuronal (TUJI) markers and reduced proliferation and self-renewal in neurospheres through the modulation of the ERK1/2 pathway [181]. Furthermore, ATRA impaired the in vitro and in vivo proliferation of CSCs by decreasing the expression of OCT4, SOX2, Nestin, and CD44 and the activation of Wnt/β-catenin signaling in head and neck cancer [182]. In lung cancer, ATRA, in combination with gefitinib, reduced the $ALDH1A1^{high}/CD44^{high}$ CSC subpopulation and growth boosted by chemotherapy [183]. Several studies have shown how ATRA's epigenetic processes work [184,185].

Although the use of NPs might be faced with some problems, such as screening difficulties, NPs are characterized by peculiar biological structures, which make them an appealing approach to anticancer therapy [186].

4. Natural Products in Clinical Trial for Cancer Treatment

The high costs and side effects of chemotherapy and radiotherapy have led to a great interest in natural medicine. NPs being readily available and more tolerable in comparison with synthetic compounds make them attractive agents for cancer treatment [187]. Many NPs, such as curcumin, EGCG, resveratrol, quercetin, and apigenin, have shown strong anticancer effects in numerous pre-clinical studies, but the feasibility of translating the efficacy of these compounds in clinical trials is an ongoing challenge. Differences in genetics and metabolism between pre-clinical models and humans, as well as the solubility and the time of action of these compounds, could limit their use in clinical settings. Pharmacokinetics and pharmacodynamics studies could better elucidate NPs' effects on humans. Despite several pre-clinical studies showing the therapeutic potential of curcumin, a few clinical trials assessed the effectiveness of curcumin in the treatment of cancer patients due to its reduced bioavailability [188]. Nonetheless, the use of curcumin as a substitute for corticosteroids (standard therapeutic agents) in combination with immunomodulatory compounds (lenalidomide) or as a proteasome inhibitor (bortezomib) in 15 multiple myeloma patients showed progression-free survival without the negative side effects linked to the steroid-based combination therapy [189]. Moreover, in multiple myeloma and prostate cancer patients, curcumin and piperine in combination delayed cancer progression (NCT04731844). The effect of curcumin on myeloma patients was also evaluated in a pilot randomized clinical trial. The treatment with melphalan, prednisone, and curcumin displayed an increased overall remission with a reduction in IL-6, VEGF, and TNF-α levels compared with myeloma patients treated with melphalan and prednisone alone [190] (ISRCTN14131419). In another clinical study enrolling 150 patients with advanced or metastatic breast cancer, the intravenous administration of curcumin (300mg) with paclitaxel (80 mg/m^2) for 12 weeks induced a tumor reduction of 50.7% [191] (NCT03072992). There are many active clinical trials to evaluate curcumin's chemopreventive, neoadjuvant, and radioprotective effects in breast cancer patients (NCT01975363, NCT03847623, NCT01246973). The phase IIa CUFOX clinical trial assessed the safety and effect of curcumin in combination with FOLFOX-based chemotherapy in metastatic colorectal cancer patients [192] (NCT01490996).

EGCG is a polyphenol with multiple antitumor activities [93]. In a second phase clinical trial, the effects of indole-3-carbinol (I3C) and EGCG in combination with taxane and platinum-based chemotherapy were tested in stage III–IV serous ovarian cancer patients.

Patients treated with I3C and EGCG plus chemotherapy showed an increased median overall survival (60 months) and median progression-free survival (48.5 months) compared with single treatment and chemotherapy alone. Moreover, I3C and EGCG treatment reduced cancer recurrence [193] (ACTRN12616000394448). In a phase II study, EGCG treatment was evaluated in chemotherapy-treated advanced lung cancer patients who had developed acute radiation esophagitis as a side effect. Both EGCG preventive treatment and EGCG administration in radiation-treated patients decreased the esophagitis grade and the serum levels of pro-inflammatory factors compared with standard treatments in lung cancer patients [194] (NCT02577393). Similarly, a phase II study evaluated the effect of EGCG in esophageal cancer patients with esophageal obstruction [195] (NCT05039983). In bladder cancer patients, a phase II randomized pre-clinical trial assessed the effect of Polyphenon E (a green tea polyphenol formulation in which EGCG is a main component) in neoadjuvant therapy before the transurethral resection of a bladder tumor or cystectomy. Although there are no differences in EGCG tumor levels between the EGCG-treated and placebo patient groups, a dose-dependent downregulation of two tumor biomarkers, clusterin (apoptosis marker) and PCNA (proliferation marker), was observed in EGCG-treated patients, supporting the chemoprotective activity of this compound [196]. The impact of Polyphenon E on serum and tissue levels of progression biomarkers was characterized in a randomized phase II single-arm open-label clinical study including breast cancer patients. In particular, Polyphenon E neoadjuvant daily administration induced a decreasein serum HGF levels without altering those of VEGF (NCT00676793). The serum HGF and VEGF levels together with the measurement of oxidative damage and inflammatory biomarkers were also evaluated in a phase IB randomized dose-escalation trial in stage I–III hormone receptor-negative breast cancer patients treated with Polyphenon E for 6 months. After the treatment with adjuvant therapy, a significant, but transient, decrease in serum HGF and VEGF was observed [197].

Although many in vitro and in vivo studies have highlighted the potential use of resveratrol in clinical settings, a limited number of clinical trials have been carried out [198]. A phase I clinical study in breast cancer found that resveratrol was tolerated throughout a 12-week treatment period on 39 patients and increased levels of resveratrol were found in blood serum patient samples. After resveratrol treatment, no significant changes in p16, CCND2, APC, orRASSF-1α DNA methylation were observed, but only a decreased methylation profile of RASSF-1α and an increase in the APC profile [199]. These findings imply that resveratrol may operate as a chemopreventive agent for breast cancer by affecting the epigenetics of breast cancer-related genes. Alternatively, in a phase I clinical study, treatment with MPX (pulverized muscadine grape skin composed ofellagic acid, quercetin, and resveratrol) in biochemically recurrent prostate cancer patients (BRPC), at different concentrations (500 mg up to 4000 mg/day), was shown to be safe and tolerable [200]. Taken together, these data result in the possibility of investigating MPX's effects in a randomized, multicenter phase II trial. In 112 patients of BRPC enrolled in a randomized, multicenter, placebo-controlled clinical trial, no significant difference was observed in terms of PSA doubling time in control and MPX-treated cohorts. Moreover, within the clinical trial, the authors identified a patient population that could benefit from treatment with MPX, but further studies are needed [201].

In another phase I pilot trial (NCT00256334), the effects of low doses of resveratrol derived from plants and resveratrol-containing freeze-dried grape powder (GP) were evaluated on Wnt-signaling modulation in colon cancer patients. Resveratrol/GP treatment (80 g/day containing 0.07 mg of resveratrol) significantly inhibited Wnt target gene expression (myc, jun, TCF7, cyclin D1, axin II) in healthy colonic mucosa without effects on tumor mucosa. This study highlights that GP treatment could have a role in the prevention of colon tumor formation [202]. In this clinical pilot study, SRT501, a micronized form of resveratrol, was administered to patients with colorectal cancer and hepatic metastases, who were scheduled to undergo hepatectomy, at a dose of 5.0 g daily for 14 days. This treatment method increased drug availability and absorption. After 1–2 weeks of therapy

with resveratrol or SRT501, the observed amounts of parent resveratrol and its primary metabolites in the colon tissue of patients were comparable to the efficacious doses of resveratrol utilized in pre-clinical investigations. In addition, cleaved caspase-3, an indication of death, dramatically increased in malignant hepatic tissue after SRT501 therapy by 39% in comparison to tissue from patients who received a placebo [203]. Furthermore, a similar first-phase clinical trial has also confirmed the diminishing of ki-67 levels (a proliferation marker) in colorectal cancer patients [204] (NCT00433576). While overall data suggest that resveratrol has some pharmacological properties, it is uncertain if these effects are sufficient to make it an effective treatment agent for colon cancer. To date, 11 of 16 marine drugs are used in the treatment of different cancers [205]. In particular, Plitidepsin (Aplidin®, produced by PharmaMar) is a drug approved in Australia for multiple myeloma leukemia and lymphoma [206]. Polatuzumabvedotin (peptide derived from marine cyanobacteria), by inhibiting tubulin polymerization, induced CSC death. This NP was approved in 2019 by the FDA for the treatment of non-Hodgkin lymphomas, chronic lymphocytic leukemia, and B-cell lymphomas [207]. Lurbinectedin (a synthetic derivative of trabectedin) showed anticancer activity by the degradation and inhibition of RNA polymerase II; in 2020, it was approved for metastatic small lung cancer treatment [208]. In this regard, other molecules derived from the marine environment are undergoing clinical evaluation.

Vincristine belongs to antimitotic agent groups that interfere with microtubule organization. Several pre-clinical studies have demonstrated its role as an anticancer agent. Nonetheless, it has been shown that vincristine provokes neurotoxicity, suggesting its use at low dosages. In several clinical trials, vincristine was used at low concentrations in combination with doxorubicin, dacarbazine, methotrexate, and also rituximab [209]. In a clinical study carried out on children affected by low-grade glioma, treatment with vincristine and carboplatin was defined as eligible first-line therapy, representing the first European clinical randomized study and the second of European chemotherapy in childhood LGG [210] (European Union Clinical Trials Register No. 2005-005377-29). In an open-label, multicentre II phase clinical trial, pretreatment with ofatumumab (antibody against CD20) and mini-CHOP (a combination with vinacristine, reduced-dose cyclophosphamide, prednisone, and doxorubicin) in 80-year-old patients improved overall survival in comparison with standard therapy [211] (NCT01195714).

Bryostatin-1 showed different effects mediated by the modulation of protein kinase PKC activity. Due to a lack of pharmacokinetics data in humans, the first clinical trials were hampered [212]. Alone or in combination with another drug, bryostatin-1 has been evaluated in phase I and II clinical trials. These multiple trials showed that this lactone, alone or in combination with other compounds, exerts synergistic anti-tumor activity. In a phase II trial, patients with metastatic renal carcinoma, treated with an intravenous infusion of bryostatin-1 with formulation PET (polyethyleneglycol, ethanol, and Tween 80), responded to the treatment without severe adverse effects [213]. The treatment of 25 patients with chronic lymphocytic leukemia (CLL) and relapsed low-grade non-Hodgkin lymphoma with bryostatin-1 resulted in one patient in complete remission and two in partial remission. Moreover, this treatment promoted a differentiative state of CLL cells, demonstrated by the presence of CD11c/CD22/CD20 B-cell subpopulation [214]. Nevertheless, Bryostatin-1 is not very available in nature, and it could need to be synthesized.

Chemicals produced from cruciferous vegetables, such as sulforaphane (SFN), a breakdown product of glucoraphanin, may help inhibit prostate cancer development and progression. A double-blind, randomized controlled trial, conducted on ninety-eight men scheduled for prostate biopsy, evaluated the effect of broccoli sprout extract (BSE) on the expression of different prostate cancer biomarkers such as histone H3 lysine 18 acetylation (H3K18ac), HDAC3, HDAC6, Ki67, p21, and histone deacetylase (HDAC). Unfortunately, BSE-treated patients did not significantly display a reduction in HDAC activity or prostate tissue biomarkers. By performing an RNA-seq analysis on prostate biopsies, 40 differently expressed genes linked to BSE treatment were characterized, including two prostate cancer-related genes, AMACR and ARLNC1. According to this study, supplementing with BSE

is associated with alterations in gene expression but not with changes in prostate tissue biomarkers [215] (NCT01265953). Furthermore, an interventional clinical trial evaluated the effect of a diet rich in broccoli in reducing the risk of cancer progression, especially in men diagnosed with low- and intermediate-risk prostate cancer on active surveillance. Trans-perineal template biopsies from forty-nine men on active surveillance, who were fed different glucoraphanin-rich broccoli soups for 12 months, were analyzed by RNA sequencing. The obtained results displayed a reduced expression of genes linked to inflammation processes and EMT in men consuming the glucoraphanin-rich broccoli diet. Although the trial lacked the necessary power to evaluate clinical progression, an inverse relationship was found between the consumption of cruciferous vegetables and a decreased risk of prostate cancer advancement [216] (NCT01950143).

In a phase I clinical study, 37 colorectal cancer patients treated with chemotherapy were randomized to receive either 100 mg fisetin (n = 18) or placebo (n = 19) for seven consecutive weeks. Fisetin administration reduced the plasma levels of IL-8, hs-CRP, and the expression of MMP-7. Accordingly, fisetin might decrease the inflammatory state in colorectal cancer patients, supporting fisetin treatment as a potential supplementary anticancer drug for these patients and warranting future investigations [217] (code: IRCT2015110511288N9) (Table 4).

Table 4. Natural products in clinical trials for cancer treatment.

NPs (Source)	Tumor Type	Phase	Patient Number	Parameters	Results	References and CT Number
Curcumin (*Curcuma longa*)	Myeloma	I	15	Patients displaying intolerance to dexamethasone treated with curcumin (3.0–4.0 g/day oral administration) plus immunomodulatory drugs (IMD, lenalidomide) or proteasome inhibitors (PI, bortezomib) for about 6 years.	Curcumin in combination with IMD or PI lessened paraprotein (38%) and plasmacytosis (59%) levels; 12 out of 15 patients were stable.	[189]
	Myeloma and prostate	II	40	Patients were treated with curcumin (4 g) plus piperidine (5 mg) by oral administration for 12 months.	No posted results. First results will be posted after May 2023.	NCT04731844
	Myeloma	IIa	33	The treated patient group (17) was treated with curcumin (8 g/day) for 28 days plus melphalan (4 mg/m^2) and prednisone (40 mg/m^2) for 7 days. Control patient group (16) was treated with melphalan, prednisone, and a placebo. The two groups received 4 cycles of treatment.	The treated group displayed an increased overall remission with a reduction in IL-6, VEGF, and TNF-α levels compared with the control group.	[190] ISRCTN14131419
	Metastatic breast cancer	II	150	Treated patient group was treated with intravenous curcumin (300 mg) and paclitaxel (80 mg/m^2) weekly for 12 weeks. Control patient groups were treated with paclitaxel and a placebo.	Tumor reduction by 50.7% in curcumin treatment compared with 33.3% placebo.	[191] NCT03072992
	Obese women characterized by high risk of developing breast cancer	I	29	The participants received curcumin (50 or 100 mg) by oral administration twice daily for 3 months.	No posted results.	NCT01975363
	Breast cancer patients before surgery	Not applicable	30	Patients were treated with 8 g per day by oral administration for two to four weeks before surgery.	No posted results.	NCT03847623
	Breast cancer patients treated with radiotherapy	II/III	686	Patients were treated with curcumin (6.0 g) by oral administration daily for the entire period of the radiation treatments plus another week.	Breast cancer patients treated with curcumin displayed a reduced dermatitis severity.	NCT01246973
	Metastatic colorectal cancer	IIa	41	Treated patient group was treated with curcumin (2 g/day) orally administered plus standard chemotherapy (FOLFOX) every 2 weeks for 12 cycles.	The clinical trial assessed the safety and effect of curcumin in combination with FOLFOX-based chemotherapy in metastatic colorectal cancer patients.	[192] NCT01490996
EGCG	Ovarian cancer	II	300	Five treatment arms: (i) Combined treatment (TP: paclitaxel 175 mg/m^2 plus cisplatin 75–100 mg/m^2 by intravenous administration, or TC: paclitaxel 175 mg/m^2 plus carboplatin AUC 5 by intravenous administration, plus surgery plus postoperative TP or TC regimen) in combination with I3C (200 mg/day) continuously; (ii) combined treatment plus I3C (200 mg/day) and EGCG (200 mg/day) continuously; (iii) combined treatment plus I3C and EGCG continuously and TP therapy; (iv) combined treatment without TP or TC postoperative regimen; (v) combined treatment.	Patients treated with I3C and EGCG plus chemotherapy showed an increased median overall survival (60 months) and median progression-free survival (48.5 months) compared with single treatment and chemotherapy alone. Moreover, I3C and EGCG treatment reduced cancer recurrence.	[193] ACTRN12616000394448

Table 4. *Cont.*

NPs (Source)	Tumor Type	Phase	Patient Number	Parameters	Results	References and CT Number
EGCG (Polyphenon E)	Lung cancer	II	83	Prophylactic EGCG group: EGCG (440 umol/L) 0.9% saline solution 3 times/day at the beginning of radiotherapy treatment; therapeutic EGCG group: EGCG 0.9% saline solution 3 times/day in presence of grade 1 esophagitis radiotherapy side effects; conventional therapy group: mLDG (lidocaine 0.16 mg/mL, dexamethasone 0.02 mg/mL, and gentamycin 0.16 mg/mL) 3 times/day in presence of grade 1 esophagitis radiotherapy side effects.	Compared to standard therapies, EGCG preventive treatment and EGCG administration in radiation-treated patients reduced the severity of esophagitis and the levels of pro-inflammatory factors in the serum.	[194] NCT02577393
	Esophageal Cancer	I	15	Six escalating doses (880 umol/L–4400 umol/L) of EGCG weredissolved in 0.9% saline solution and administered three times a day. EGCG solution was given continuously for 8 days before anti-tumor treatment.	No posted results (ongoing trial).	[195] NCT05039983
	Bladder cancer	II	31	An amount of 800–1200 mg/day of orally administered EGCG for 14–28 days prior to surgery.	The dose-dependent downregulation of two tumor biomarkers, clusterin (an apoptosis marker) and PCNA (a proliferation marker), was seen in EGCG-treated patients, supporting the compound's chemoprotective activity and use as a neoadjuvant therapy before transurethral resection of bladder tumor or cystectomy, despite the fact that there are no differences in EGCG tumor levels between EGCG-treated and placebo patient groups.	[196]
	Breast cancer	II	32	Subjects are asked to take 4 polyphenol E (200 mg) capsules daily with a meal for the duration of the study. Biomarkers are measured at baseline and then again at presurgery, the end-point for the study within a time frame between 4 and 6 weeks.	Polyphenon E neoadjuvant daily administration induced a decreasein serum HGF levels, without altering those of VEGF.	NCT00676793
	Breast cancer	Ib	40	After completing adjuvant therapy, women with stage I–III breast cancer were randomized to receive Poly E at dosages of 400, 600, or 800 mg twice daily for six months, or a placebo. Samples of blood and urine were collected at the beginning, 2, 4, and 6 months.	After completing adjuvant therapy, women with stage I–III breast cancer were randomized to receive Poly E at dosages of 400, 600, or 800 mg twice daily for six months, or a placebo. Samples of blood and urine were collected at the beginning, 2, 4, and 6 months.	[197]
Resveratrol	Breast cancer	I	39	Resveratrol (5–50 mg) was orally administered for 3 months, twice a day.	After resveratrol treatment, no significant changes in p16, CCND2, APC, and RASSF-1α DNA methylation wereobserved, but only a decreased methylation profile of RASSF-1α and an increase inthe APC profile.	[199]
	Prostate cancer	I	14	Patients were treated with 500 to 4000 mg of muscadine grape extract (MPX, containing 1.2 mg of ellagic acid, 9.2 g of quercetin, and 4.4 g of trans-resveratrol) per day, taken orally for 28 days, with a follow-up of >2 years.	No tolerability problems were observed in patients, and the treatment was deemed to be safe. Additionally, it demonstrated a delay in the recurrence process by extending the PSA doubling time (PSADT) by 5.3 months.	[200]
	Prostate cancer	I	112	Following stratification based on their initial PSADT and Gleason scores, the participants were randomly allocated 1:2:2 to receive a placebo, 500 mg of MPX (low), or 4000 mg of MPX (high), daily.	There was no discernible difference between the control and MPX-treated cohorts in the time it took for PSA to double.	[201] NCT00256334
	Colorectal cancer	I	8	Resveratrol was administered at 20 and 80 mg/day. Resveratrol-containing freeze-dried grape powder (GP) was administered at 0.073 and 0.114 mg/day. Both treatments were taken orally.	Resveratrol/GP treatment significantly inhibited Wnt target gene expression (myc, jun, TCF7, cyclin D1, axin II) in healthy colonic mucosa without effects on tumor mucosa. This treatment method increased drug availability and absorption.	[202]
	Colorectal cancer	I	9	A 5.0 g daily dose for 14 days of SRT501 (micronized form of resveratrol), was administered to patients with colorectal cancer and hepatic metastases who were scheduled to undergo hepatectomy.	After 1–2 weeks of therapy with resveratrol or SRT501, the observed amounts of parent resveratrol and its primary metabolites in the colon tissue of patients were comparable to the efficacious doses of resveratrol utilized in pre-clinical investigations. In addition, cleaved caspase-3, an indication of death, dramatically increased in malignant hepatic tissue after SRT501 therapy by 39% in comparison to tissue from patients who received a placebo.	[203]
	Colorectal cancer	I	20	Colorectal cancer patients were treated daily with resveratrol (0.5 or 1.0 g) for 8 days before surgery.	Treatment reduced ki-67 levels.	[204] NCT00433576

Table 4. *Cont.*

NPs (Source)	Tumor Type	Phase	Patient Number	Parameters	Results	References and CT Number
Vincristine	Low-grade glioma	I	497	A total of 497 patients were randomized to receive vincristine carboplatin (VC, vincristine 1.5 mg/m^2 × 10 weekly and carboplatin 550 mg/m^2 q 3 weekly) (n = 249) or VC plus etoposide (VCE, etoposide 100 mg/m^2, days 1, 2,and 3).	The high rates of non-progression after 24 weeks support the use of VC as a first-line treatment.	[210] European Union Clinical Trials Register No. 2005-005377-29
	Diffuse large B-cell lymphoma	II	120	Patients underwent a pre-treatment phase consisting of oral prednisone (60 mg total dosage commencing 1 week before cycle 1, for 4 days (day7 to day4)) and oral vincristine before the first cycle of the ofatumumab (1000 mg every 3 weeks) + miniCHOP regimen (400 mg/m^2 of intravenous cyclophosphamide, 25 mg/m^2 of intravenous doxorubicin, 1 mg/m^2 of intravenous vincristine on day 1 of each cycle, and 40 mg/m^2 of oral prednisone every day from days 1 to 5).	The pretreatment with ofatumumab and miniCHOP in 80-year-old patients improved overall survival in comparison with standard therapy.	[211] NCT001195714
Bryostatin-1	Renal cell carcinoma	II	30	Patients were treated for 30 min with an intravenous infusion of bryostatin-1 (25 microg/m^2) with formulation PET (polyethyleneglycol, ethanol, and Tween 80) on days 1, 8, and 15 of each 28-day cycle.	Patients responded to the treatment without severe adverse effects.	[213]
	Low-grade non-Hodgkin lymphoma and chronic lymphocytic leukemia	II	25	Patients were treated for 72 h with a continuous infusion of bryostatin-1 (120 microg/m^2) per course every 2 weeks immediately followed by vincristine (from 0.5 mg/m^2 to 2 mg/m^2) administration by bolus i.v. injection.	Treatment with bryostatin-1 resulted in one patient in complete remission and two in partial remission. Moreover, this treatment promoted a differentiative state of CLL cells, demonstrated bythe presence of CD11c/CD22/CD20 B-cell subpopulations.	[214]
Sulforaphane	Prostate cancer	nd	98	Patients were treated with BSE (200 µmol daily) or a placebo for 4–8 weeks.	Forty differently expressed genes linked to BSE treatment, including the downregulation of two prostate cancer-related genes. Supplementing with BSE is associated with alterations in gene expression but not with changes in prostate tissue biomarkers.	[215] (NCT01265953)
	Prostate cancer	II	61	Patients were given a weekly 300 mL serving of soup produced from either regular broccoli (the control) or one of two experimental broccoli genotypes with increased glucoraphanin concentrations that delivered 3 or 7 times the amount of the control, respectively.	Downregulation of genes linked to inflammation processes and epithelial–mesenchymal transition in a dose-dependent manner in glucoraphanin-rich broccoli-soup-consuming men. An inverse relationship was found between the consumption of cruciferous vegetables and a decreased risk of prostate cancer advancement in males under active monitoring.	[216] (NCT01950143)
Fisetin	Colorectal cancer	I	38	CRC patients treated with chemotherapy were randomized to receive either 100 mg fisetin (n = 18) or placebo (n = 19) for 7 weeks.	After fisetin administration to CRC patients, plasma levels of IL-8 and hs-CRP dropped dramatically as a lower expression of MMP-7.	[217] (code: IRCT2015110511288N9)

Abbreviations: IMD, immunomodulatory drugs, PI, proteasome inhibitor, VEGF, vascular endothelial growth factor, IL-6, interleukine-6, TNF-α, tumor necrosis factor-α, FOLFOX, leucovorin calcium (folinic acid), fluorouracil, and oxaliplatin., EGCG, epigallocatechin-3-gallate, TC, taxotere and cyclophosphamide, TP, docetaxel plus cisplatin, PCNA, proliferating cell nuclear antigen, HGF, hepatocyte growth factor, CCND2, Cyclin D2, APC, WNT signaling pathway regulator, RASSF-1α, Ras association domain-containing protein 1 I, TCF7, Transcription Factor 7, SRT501, micronized form of resveratrol, miniCHOP, mini-cyclophosphamide, doxorubicin, vincristine, and prednisone, CLL, chronic lymphocytic leukemia BSE, broccoli sprout extract, CRC, colorectal cancer.

5. Conclusions and Perspectives

In recent years, the rising costs of cancer treatment and the urgent need for eco-sustainability endorse a new paradigm in oncology, known by the term "Green Oncology". To date, the ecological model, in which oncologists consider not only individual illness but also population health as a component of the biosphere, is increasingly replacing the biomedical and biopsychosocial ones. In this context, Green Oncology's aim is to preserve the environment and the ecosystem by promoting the use of NP-derived drugs, which avoids treatments with chemotherapeutics that are not easily disposable and are also characterized by fewer side effects. Compelling evidence points out that NPs effectively lessen the stem-like properties of CSCs, which are refractory to standard and targeted

therapies. In this review, we reported the most appealing pre-clinical studies regarding the ability of NPs to lessen the expression of CSC markers and the activation of pro-tumorigenic signaling pathways. Despite the promising results obtained with the use of NPs in in vitro systems and pre-clinical models, the limited systemic availability of these all-natural molecules as well as their faster metabolism pose a challenge to their efficacy in targeting cancer cells in organs far from the site of absorption. Although NPs could really improve the malignant progression of tumors, further efforts are needed to reduce the timeline of bench to bedside.

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Chapter 2
Humic substances enhance anti-cancer efficacy of standard therapies

Humic substances enhance anti-cancer efficacy of standard therapies

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Abstract

The Green Oncology paradigm highlights the potential of using natural products in cancer treatment to safeguard the ecosystem and reduce the side effects associated with conventional drugs. Within this framework, humic substances (HS) derived from waste biomass degradation have demonstrated promising properties. Here, we demonstrated the antioxidant, antimicrobial, and antitumor effects of HSs derived from olive (HS-OL) and artichoke (HS-CYN). Our findings showed that HS-OL and HS-CYN effectively reduced cell viability and induced DNA damage, leading to apoptosis in colorectal, thyroid, and breast cancer cells. By enhancing standard therapies, HS-OL and HS-CYN can provide optimal clinical benefits while allowing for lower concentrations of chemotherapeutic agents. In conclusion, incorporating HSs into cancer treatment represents an eco-friendly therapeutic strategy that promotes the recycling of plant biomass and minimizes the side effects associated with high doses of anticancer drugs.

Keywords: humic substances, artichoke, olive, cancer stem cells, colorectal cancer, thyroid cancer, breast cancer.

Introduction

Green oncology introduces an innovative approach to cancer treatment, focusing on tailoring specific therapies to individual patients. This strategy not only focuses on the efficacy of treatments but also emphasizes their social, economic, and environmental sustainability. By integrating these dimensions, green oncology aims to enhance patient care while ensuring that treatment options are viable and responsible within broader societal contexts[1]. In 2019, there were approximately 23.6 million new cancer cases diagnosed globally, resulting in an estimated 10 million cancer-related deaths and around 250 million patients experiencing disabilities following treatment. According to the IQVIA Global Oncology Trends 2021 report, oncology drug sales reached USD 164 billion in 2020, reflecting an average annual growth rate of 14.3% over the preceding five years[2]. First-line treatment for solid tumors typically involves a combination of surgery, chemotherapy, and radiotherapy. While these approaches have improved efficacy and patient survival rates, they impose substantial costs on public health systems. Moreover, short and long-term side effects of these treatment regimens pose significant challenges for patient care and clinicians. To address these concerns, researchers have been developing new strategies that improve treatment tolerance and optimize the scheduling of radiotherapy or chemotherapy. In this context, natural products could serve as promising candidates for successful alternative therapies. Natural bioactive compounds retain unique properties related to their structure, high chemical diversity, and low toxicity, making them suitable for several biomedical applications[3]. Humic substances (HS), derived from the decomposition of organic matter, exhibit biological activity that significantly impacts soil health, enhances plant growth, and facilitates microbial interactions[4]. The effects of HSs on human health have garnered increasing scientific attention due to their potential therapeutic advantage[5]. As the main component of HSs, humic acid possesses antioxidant, anti-inflammatory, and immune-modulating properties by regulating cytokine production, which is essential for managing inflammation and autoimmunity [6,7,8]. It effectively mitigates oxidative stress by scavenging free radicals, thus protecting cells from oxidative damage and potentially slowing the aging process[9,10]. The regenerative properties of HS are noteworthy in the field of dermatology and tissue engineering, as their incorporation into biomaterials could enhance the integration and performance of tissue scaffolds[39-42]. In cancer research, HSs exhibited potential anti-cancer properties by inducing apoptosis and inhibiting the proliferation of various cancer cell lines[11,12]. These effects are likely attributed to its interactions with cellular signaling pathways that regulate cell growth and survival. Despite these promising findings, it is important to note that the biological activity of humic acid may depend on its source, composition, and concentration[43]. Therefore, rigorous standardization and quality control are essential in research and therapeutic applications.

Here, we investigated the anti-tumor potential of HSs derived from the decomposition of olive and artichoke (HS-OL and HS-CYN respectively), on different cancer cells. The biological activity of both HS-OL and HS-CYN is primarily attributed to their high content of bioactive chemical groups, particularly polyphenols such as oleuropein and hydroxytyrosol. These compounds exhibited antioxidant, anti-inflammatory, and immunomodulatory properties[13,14].

We investigated the effectiveness of HS-OL and HS-CYN as adjuvant therapy in three tumor models: colorectal cancer patient-derived spheres (CR.CSC#2 and #9[15]), anaplastic thyroid cancer cell lines (ATC: Cal62 and 8505c), and breast cancer cells (MDA-MB-231 and breast cancer sphere cells, BCSphCs#21[16]). Interestingly, treatment with HS-OL and HS-CYN significantly impaired cell viability and increased apoptosis in all the cancer models analyzed. Additionally, both natural compounds induced DNA damage, sensitizing these aggressive cancer cells to chemo-radiotherapy and enhancing the overall anti-tumor activity of the standard treatments. These findings indicate that HS-OL and HS-CYN could be valuable adjuvants to standard treatment protocols for patients with advanced tumors.

Results

HS display antioxidant and antibacterial activities.

The chemical characterization of HSs was analyzed using solid-state 13C CPMAS-NMR. The NMR spectra for HS-OL and HS-CYN (**Figure 1A**) revealed a significant presence of aliphatic and aromatic apolar fractions, which constitute about 40% of the total area (**Figure 1B**). In olive-derived samples, the alkyl-C chemical range represented up to 22% of the total area. Artichoke-derived humic products showed approximately 24.1%, 23.6%, and 14.3% of the total carbon signals, respectively. A contrasting trend was noted in the distribution of aromatic moieties. HSs from artichoke wastes contained aryl and O-aryl-C components that made up 18.7% and 5.3% of the total signal area, respectively. In contrast, olive-derived humic substances exhibited a lower content of 14.3%. Both HSs are marked by intense peaks of methoxyl groups linked to aromatic rings at 55/56 ppm, indicating effective incorporation of lignin monomers. The results highlighted a predominance of O-alkyl carbon in the region between 60-110 ppm, typically associated with oligo and polysaccharides. The hydrophobicity index (HB) data demonstrated a balanced distribution between apolar compounds and polar fractions. The alkyl ratio (A/OA) suggested a predominance of apolar aliphatic groups in HS-OL pomace residues, likely due to the high content of fatty acids and bio-polyesters in these plant residues. The aromaticity indices were found to be equal to 0.5% for each type of HS, as shown in Figure 1B. The lignin ratio (LigR) values ranged from 3.1 to 2.8 for HS-OL and HS-CYN,

respectively, confirming the correlation between methoxyl group signals between 45 and 60 ppm and phenolic carbons in the spectral range between 140 and 160 ppm. This supports the hypothesis that lignin monomers are incorporated into the aromatic fractions of compost and humic extracts. We observed that the ABTS assay revealed higher percentage inhibition values for compost extracts from olive compared to those from artichoke (**Figure 1C**). Specifically, HS-OL showed 73% inhibition, while HS-CYN exhibited 64%. When expressing the ABTS antioxidants as TEAC (mmol Trolox equivalent per kg of the sample), HS-OL had a value of 408.5, followed by HS-CYN with 380. This comparison highlights the antioxidant capacity of the humic extracts against a standard antioxidant compound. A similar trend was noted in the FRAP assay results (**Figure 1D**), which are expressed as mg Trolox equivalents per gram of sample. This spectrophotometric determination confirmed that HS-OL had a greater antioxidant capacity than HS-CYN, with values of 254 and 127.5, respectively. Consistent with previous studies by [5,7,17], the antioxidant activity of HS extracts from green composts correlated directly with their total phenolic content (TPC), as measured by the Folin-Ciocalteu assay (**Figure 1E**). The phenolic composition was also significant, with percentages of 60% and 40%, respectively, as shown in **Figure 1F**. Although the Folin-Ciocalteu reagent is not specific for polyphenols, our findings align with previous research suggesting a strong correlation between TPC and antioxidant capacity in green compost extracts. This correlation is crucial for developing novel and sustainable antioxidant systems. Moreover, the compounds exhibiting greater antioxidant and antibacterial activity were consistent across samples and showed high total phenol content, with values of 156 and 103 GAE (mmol gallic acid equivalents per mg sample) for HS-OL and HS-CYN, respectively (**Figures 1E and F**).

Bacterial infections represent a direct threat to human health due to progressive drug resistance and the emergence of new pathogenic strains[18]. Natural products provide a specific source of bioactive metabolites, which can be exploited for their therapeutic effects[19,7,17,20,21]. In this work, we evaluated the antimicrobial efficacy of HSs derived from artichoke and olive tree composted wastes. The method for assessing the potential antibacterial activity of these green extracts was that of the diffusion disk (DDK)[5]. In our screening HSs derived from artichoke or olive compost were tested against certain Gram-positive (*S. epidermis* and *L. monocitogenes*) and Gram-negative bacterial strains (*H. pylori* and *S.thyphi*), while a protein such as bovine serum albumin (BSA) or a common antibiotic such as ampicillin, was used as a negative or positive reference control, respectively. Based on the analysis of all the extracts tested, a strong antibacterial activity was observed against Gram-positive bacterial strains defined as multi-drug resistant such as *S. epidermis with* an inhibition halo of 9.4 mm for HS from olive trees while 6.3 in the case of HS from artichoke (Figure 1G). In addition, clear antimicrobial activity has been exhibited from HS-OL against *H.pylori* and *S.thypi*,

two different microbial strains involved in the pro-tumoral inflammation microenvironment in gastric cancer[22]. Our work is the first example of HSs' application, in the impairment of human microbial patogen proliferation. In this context, our results suggest that the bioactivity of organic extracts from composted waste biomass is strongly related to the molecular composition of organic extracts and the abundance of aromatic and phenolic fractions in these products.

Exposure to HS-OL and HS-CYN affect cancer cell survival

The anti-tumor effects of HS-OL and HS-CYN were examined in three distinct models, specifically colorectal cancer, ATC, and breast cancer. In our study, we investigated the sensitivity of CR-CSCs (CR-CSC #2 and CR-CSC#9) to the treatment with HS-OL and HS-CYN. These CR-CSCs represent a critical subpopulation within tumors, significantly contributing to tumor initiation, metastasis and resistance to treatment[23,24]. Additionally, we examined-two cellular types (8505c and Cal62) derived from ATC and other from breast tumors (BCSphCs#21 and MDA-MB-231), characterized by their rapid growth[25]. CR-CSphCs and BCSphCs are part of our extensive collection of primary cell lines that faithfully represent the genomic and transcriptomic profiles of the original cancer cells[16,25]. Treatment with HS-OL and HS-CYN resulted in a 50% reduction in cell viability within 24 hours for colorectal, thyorid and breast cancer cells (**Figure 2A, B and Figure S1A, B**). In contrast to their effects on cancer cells, HS-OL and HS-CYN had only a slight impact on non-tumorigenic human cell lines such as HUVEC and IMEC (**Figure S1C**). Both the cell lines serve as effective models for studying the toxic effects of drugs[23]. The ability of HS-OL and HS-CYN to significantly reduce the viability of aggressive cancer cells while minimally affecting healthy cells suggests that these compounds could be valuable additions to cancer treatment strategies.

HS-OL and HS-CYN induce DNA damage

We proposed that the anti-cancer effects of HSs might be related to changes in cell cycle progression and the induction of apoptosis. When exposed to HS-OL and HS-CYN, colorectal, thyroid, and breast cancer cells showed an increased fraction of cells in the sub-G0 phase (**Figure 2C**). Consequently, there was an observed increment of Caspase-3 activity following treatment with these HS factors in the cell lines described above (**Figure 2D, E, and S1D**). The interplay between cell cycle arrest and apoptosis is often initiated by DNA damage. By coordinating these processes, cells can effectively cope with genotoxic stress, preventing the replication of damaged DNA. Upon encountering genotoxic stress, cells activate checkpoint mechanisms that pause the cell cycle, primarily at the G1/S or G2/M transitions. This stop provides time for the cell to decide whether to repair the damage or

proceed with programmed cell death. Our study found a significant overexpression of genes related to DNA repair in colorectal, thyroid, and breast cancer cells treated with HS-OL and HS-CYN for 48 hours (**Figure 3A**). Transcriptomic analysis identified 11 genes that were upregulated across all cancer cells analyzed treated with both compounds (**Figure 3B**). It is well-known that various biological substances, along with chemical and physical factors, can cause double-strand breaks in DNA. These breaks activate specific pathways known as the DNA damage response (DDR), marked by the phosphorylation of the histone variant H2AX following cell cycle arrest and checkpoint protein activation. Our results showed that treatment with HS-OL and HS-CYN significantly increased H2AX phosphorylation in colorectal, thyroid, and breast cancer cells (**Figure 3C, D**). Overall, these findings suggested that the DNA damage induced by HS-OL and HS-CYN was substantial enough to overwhelm the cancer cells' repair mechanisms, leading to the activation of apoptotic pathways. This indicates that HSs could induce DNA damage, sensitizing cancer cells to the anti-tumor effects of standard therapeutic regimens such as radio- or chemotherapy.

HS-OL and HS-CYN sensitize cancer cells to conventional therapies

Chemotherapy and radiotherapy are the primary treatment options for cancer patients. However, aggressive cancer cells develop resistance to these standard therapies, often leading to tumor recurrence. Additionally, the side effects associated with chemotherapy can significantly reduce patients' quality of life, underscoring the urgent need for new, more effective, and better-tolerated therapeutic strategies. We sought to investigate whether HS-OL or HS-CYN could enhance the anti-tumor effects of standard chemotherapeutic agents on colorectal, thyroid, and breast cancer cells. By maximizing the anti-tumor response while using lower drug doses, this approach seeks to mitigate the side effects commonly associated with higher chemotherapy dosages. By combining a lower and less toxic dose of FOLFOX and FOLFIRI with either HS-OL or HS-CYN, we achieved anti-tumor activity comparable to that of four times the concentration of the standard therapy alone **(Figure 4A and Figure S2A)**. Moreover, the presence of HS-OL or HS-CYN dramatically reduced the viability of irradiated ATC cells (5 Gy) **(Figure 4A)**. Similarly, the highest anti-tumoral effect was obtained when doxorubicin was applied in combination with HSs in both ATC and breast cancer cells **(Figure 4A and Figure S2B, C)**. Notably, Chou–Talalay analysis demonstrated that both HS-OL and HS-CYN exhibited a synergistic effect when combined with standard treatments across all tumor models analyzed **(Figure 4B).** Therefore, a notable increment of caspase-3 activation was observed when cancer cells were treated with the two drugs in combination **(Figure S2D-F).**

These findings highlighted the promise of HS-OL or HS-CYN as a sustainable and effective compound to use in adjuvant approach alongside standard radio-chemotherapy for advanced colorectal, thyroid and breast cancer treatment.

Discussion

Despite the global scientific community's efforts to develop new therapeutic strategies, metastatic disease remains the leading cause of death among cancer patients due to its complex nature and the resistance of metastatic cells to current treatments, which often involve multiple modalities[26-28]. Additionally, the side effects of chemotherapy and radiation can severely affect patients' quality of life. Modern drug development increasingly utilizes natural compounds for various applications, including disease prevention and as antioxidants in metabolic processes[29,30,31]. Here, we demonstrated the potential anti-tumor effects of HS-OL and HS-CYN, which are derived from the decomposition of natural products thereby encompassing the concept of recycling substances to safeguard the entire ecosystem. HS-OL and HS-CYN are easily extractable from natural sources and could offer cost-saving and environmentally friendly therapeutic options. The diverse composition of HSs provides structural features that influence their bioavailability, bioactive properties, and pharmaceutical applications[32,33]. The phenolic and quinone components in HS-OL and HS-CYN extracts can neutralize free radicals, offering antioxidant benefits for both environmental and medical uses. It is suggested that phenolics interact with enzyme active sites, causing irreversible changes in cell membrane permeability or integrity, leading to bacterial cell death. Notably, Gram-positive bacteria tend to be more susceptible to polyphenols than Gram-negative bacteria due to differences in cell wall structure. Our results showed the greatest antibacterial performance of extracts from HS-OL, due to its high abundance of unfragmented polyphenols conserved.

Our findings provide evidence that HS-OL and HS-CYN have significant implications for cancer treatment, particularly against aggressive cancer types. HS-OL and HS-CYN anti-tumor activity is attributed to DNA damage induction in cancer cells, leading to cell cycle arrest and apoptosis. This process is essential for halting cancer cell proliferation and initiating programmed cell death, thereby reducing tumor burden.

When combined with standard radio/chemotherapy, HS-OL and HS-CYN significantly enhanced treatment efficacy by targeting resilient cancer cell populations. This suggests a synergistic effect that could improve patient outcomes when used alongside conventional therapies. These insights reinforce the potential of natural compounds like HS-OL and HS-CYN in developing innovative adjuvant anti-cancer therapies that are both effective and sustainable.

MATERIALS AND METHODS

Green composts and humic substances extraction Artichoke agricultural wastes were collected in composting center of the Experimental Farm of the University of Naples Federico II at Castel Volturno, Italy (as reported in Verrillo et al. 2021 a,b).[7]). Conversely, compost from olive tree residues (pomace and leaves) was produced by the CNR-ISAFOM in Perugia through a prototype composting plant as described in[34]. The prototype comprises two 30L bioreactors capable of controlling a 'closed vessel' system for composting, although it takes place as an 'industrial' type process with a typical sequence of mesophilic-thermophilic phases. Extraction of humic substances from artichoke or olive wastes (HS-CYNOL and HS- OLCYN) has been performed according to the method discussed by Verrillo et al. 2022.2022[5,17]. All humic substances products were freeze-dried before analysis.

^{13}C Crosspolarization magicanglespinning NMR spectroscopy

Solid-state ^{13}C Cross-Polarization Magic-Angle-Spinning (CPMAS) NMR Spectroscopy spectra of humic substances were performed on a Bruker AV-300, equipped with a 4 mm MAS probe as described in[7]. Generally, signal resonances are collected into six main spectral regions related to several types of carbon functional moieties.

Antioxidant features

Since the heterogenic and complexity of humic products, antioxidant activity is better evaluated by applying two complementary spectrophotometric methods[35]. Here, 2,20 -azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and Ferric reducing ability of plasma (FRAP) were employed to evaluate the antioxidant features of HS-OL and HS- CYN. Briefly, ABTS assay has been carried out as described in Verrillo et al., 2021 a.[7]. conversely, FRAP determination was performed using a FRAP reagent prepared before each measurement by mixing acetate buffer (300 mM), TPTZ (10 mM), and $FeCl_3$ (20 mM in Milli Q Water) and incubated at 37 °C for 10 min. For the analysis, 2 mL of FRAP solution was mixed with sample measuring the absorbance at 593 nm. A calibration curve with six Trolox standards was employed[36].

Antimicrobial assay

Assessment of antimicrobial capacities of humic product has been performed by diffusion disk test (National Committee for Clinical Laboratory Standards (NCCLS) method). Microbial cells include *Helicobacter pylori* (Hp), *Salmonella typhi ATCC14028* (S.T.), *Staphylococcus epidermis* (S.E.) and *Listeria monocitogenes* ATCC19115 (L.M.). The inoculum of each microbial cell was carried out by

in nutrient agar with sterile saline buffer up to 10^8 CFU/mL (0.5 McFarland). Then, 300 µL of each microorganism was placed on Mueller–Hinton agar. Finally, six disks (6.0 mm diameter) were treated with 20 µg of each humic substance, placed on the agar, and incubated at 37 °C for 24 h. Ampicillin (AMP), Clavulanic Acid and Bovine serum albumin (BSA) were employed as negative control and positive reference, respectively.

Cell culture

CR-CSC#2 and CR-CSC#9 and BCSphCs#21 were obtained from surgical tumor tissue provided by the University Hospital "P. Giaccone" and the Hospital "Ospedali Riuniti Villa Sofia-Cervello", in accordance with the ethical standards of the Institutional Committee responsible for human experimentation (authorization CE9/2015). CR-CSC and B-CSph cell purification and propagation was assessed as previously described[16,37] and DNA profiles were matched with their relative patient tumor tissues. BCSphCs#21 cells were isolated from a Her2-enriched tumor specimen, which was serially transplanted in immunocompromised mice, and displayed an enrichment in the stem cells compartment (CD44high/CD24low). Cal62, 8505, MDAMB231, Huvec and IMEC cells were obtained from ATCC/LGC Standards S.r.l. (Sesto San Giovanni, Italy), and cultured as suggested by the supplier. Mycoplasma presence was assessed with the MycoAlertTM Plus Mycoplasma Detection Kit (Lonza, Houston, TX, USA). Routinely was performed cells authentication using a short tandem repeat DNA profiling kit (GlobalFiler™ PCR kit, Applied Biosystem) following the manufacturer's instructions and analyzed by ABI PRISM 3130 (Applied Biosystem).

Cell viability

For cell viability CS-CRC#2, CS-CRC#9, Cal62, 8505c, MDA-MB-231, BCSphCs#21, Huvec and IMEC were seeded in the appropriate culture medium and treated with 0, 15, 32, 64, 125, 250, and 500 µg/mL of HS-OL or HS-CYN for 48hs to determine the IC50. Following experiments were performed with 500 µg/mL of HSs in CS-CRC#2, CS-CRC#9, Cal62 and 8505c cells, while MDA-MB-231 and BCSphCs#21cells were treated with 350 µg/mL of HS-OL or HS-CYN. CS-CRC#2 and CS-CRC#9 were treated with 1.25 µM FOLFOX (5-fluorouracil, Leucovorin and oxaliplatin), FOLFIRI (5-fluoracil, leucoverin irinotecan), respectively, alone or in combination with HS-OL or HS-CYN (500 µg/ml) for 48 hs. MDA-MB-231 and BCSphCs#21cells were treated with doxorubicin (50 nM- 400 nM range, Sigma-Aldrich) in presemce or not of HSs (350 µg/mL). Cell viability was assessed by CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega Madison, WI, USA,) according to the manufacturer‟s instructions and analyzed by Infinite® F500 (Tecan). Data from growth-inhibition assays were plotted using the nonlinear regression curve fit modeling from

GraphPad Prism-5 (GraphPad Software). Cal62 and 8505c cells, pre-treated with HS-OL or HS-CYN (500 μg/mL) for 48 hours, were irradiated at room temperature with (5 Gy) at room temperature. Following irradiation, cells were seeded in complete culture medium in 6-well plates (20,000 cells/well) with or without the addition of HS-OL or HS-CYN (500 μg/mL), which was replenished every 48 hours. After 21 days, the cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet-methanol solution (Sigma-Aldrich). For long-term proliferation assays, 8505c cells were seeded in 6-well plates (20,000 cells/well) and cultured in the absence and presence of HS-OL or HS-CYN (500 μg/mL) and doxorubicin (200 nM) alone or in combination. The wells were fixed with 4% paraformaldehyde and stained with 0,1% crystal violet-methanol solution after 14 days. Cell proliferation was quantified using ImageJ software. All assays were performed independently at least two times.

Flow cytometry analysis

For cell cycle analysis, CS-CRC#2, CS-CRC#9, Cal62, 8505c, MDA-MB-231, BCSphCs#21 treated as indicated were washed with PBS and centrifuged at 1300 rpm for 5 min. The cell pellet was resuspended in 1 mL of Nicoletti Buffer, which consisted of 0.1% sodium citrate, 0.01% Triton X-100, 50 μg/mL propidium iodide, and 10 μg/mL RNase solution. The resuspended cells were incubated in the dark at 4°C for 16 hours. Apoptotic cells were detected by using the CaspGlow Fluorescein, Active Caspase 3 Staining kit (Biovision, Milpitas, CA, USA) according to the manufacturer's protocol.

FACSLyric flow cytometer (BD Biosciences, Franklin Lakes, NY, USA was used for cell detection and FlowJo software was used for the analysis.

Protein and gene expression analysis

CS-CRC#2, CS-CRC#9, Cal62, 8505c, MDA-MB-231, BCSphCs#21 cells were harvested by scraping in ice-cold PBS and resuspended in ice-cold F buffer (Tris-HCL 10 mM, NaCl 50 mM, sodium pyruvate 30 mM, NaF 50 nM, ZnCl2 5 μM, triton 1, sodium orthovanadate 0.1 nM, sodium butyrate 10 mM and PMSF 1 mM) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Whole-cell lysates were loaded in sodium dodecyl sulfate-polyacrylamide-gel electrophoresis gels and blotted on nitrocellulose membranes. Membranes were blocked with a 5% nonfat dry milk and 0.1% Tween 20 PBS solution for 1 h at room temperature and then incubated with specific antibodies against γ-H2AX (Ser139, 20E3, Rabbit IgG, Cell Signaling Technology) and

β-actin (8H10D10, mouse IgG2b, Cell Signaling Technology). Primary antibodies were revealed using antimouse or antirabbit HRP-conjugated (goat IgG; Thermo Fisher Scientific) and detected by Amersham imager 600 (GE Healthcare). Protein levels were normalized with β-actin and calculated by densitometric analysis using ImageJ software.

RNA Isolation and Gene Expression Analysis

The purification of total RNA was obtained using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) and RNA concentration was determined with NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). For gene expression analysis, 1 µg of RNA was retrotranscribed with a PrimePCR custom panel (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions and analyzed for genes involved in DNA damage signaling pathway using a PrimePCR designed panel (Bio-Rad).

Drug Combination Study

Drug combination studies have been assessed by using the Chou–Talalay method, which is based on the median effect and the combination index (CI) equations to determine the quantization of drug interactions. The CI), computed in CompuSyn using the Chou–Talalay method, calculated on CR-cancer cell proliferation following the treatment with different concentrations of FOLFOX or FOLFIRI (1.25, 2.5 and 5µM) and different concentrations HS (125, 250, 500µg/mL). The synergism effect was calculated similarly in ATC and TNBC cells treated with different concentration of HS-OL and HS-CYN (250,350,500 µg/mL) and different concentration of doxorubicin (400 ,800, 1000 nM). CI < 1 represented synergism (slight, moderate, strong, very strong); otherwise, it indicated additivity (CI = 1) or antagonism (CI > 1) between two drugs. This combination study assessed breast cancer cells treated with humic substance and doxorubicin.

Immunofluorescence

Immunofluorescence analysis on tumor cells treated as described was performed as reported[38]. Staining was carried out with anti-cleaved-3-caspase primary antibody (ASP175, rabbit cell signaling technology) revealed by Alexa Fluor 488-conjugated secondary antibody; anti-Y-H2AX p-Histone H2AX primary antibody (S139, rabbit cell signaling technology) revealed by Alexa Fluor 555-conjugated secondary antibody. Y-H2AX positive cells were counted with ImageJ Cell Counting software, Nuclei were counterstained using Hoechest (Thermofisher).

Statistical analysis

Data were shown as mean ± standard deviation. Statistical significance was estimated by unpaired t-test. Results were referred to statistically significant as $p<0.05$. $*$ indicates $p< 0.05$, $**$ indicate $p < 0.01$, $***$ indicate $p < 0.001$, and $****$ indicate $p < 0.0001$.

Figure Legend

Figure 1: HS-OL and HS-CYN characterization. a) 13C CPMAS NMR spectra of different humic substances; **b)** 13C CPMAS-NMR spectroscopy characterization of HS-CYN and HS-OL composted wastes as relative abundance (%) of main C structures over chemical shift regions (ppm) and structural indices. a HB/HI = hydrophobicity index = [Σ(0–45) + (45–60)/2 + (110–160)/Σ(0–45) + (45–60)/2 + (60–110) + (160–190)]; A/OA = alkyl/O-alkyl ratio = [(0–45)/(60–110)]; ARM = aromaticity index [(110–160)/Σ(0–45) + (60–110)]; LigR = Lignin ratio [(45–60)/(145–160 **c, d)** Antioxidant capacity of different humic substances measured applied ABTS (p value= 0.0095) and FRAP spectrophotometric methods (p value <0.0001). **e, f)** correlation between anti-oxidants activity and phenolic content (E) and phenolic composition (F); **g)** Antimicrobial performance by diffusion disk assay of HS against two Gram positive (S. epidermis and L. monocitogenes) and two Gram negative (H.pylori and S.thypi) bacterial strains involved in human diseases.

Figure 2: HS-OL and HS-CYN treatment hamper cancer proliferation and induce apoptosis. a) Cell viability analysis of colorectal (CS-CRC#2, CS-CRC#9), thyroid (Cal62, 8505c), and breast (MDA-MB-231, BCSphCs#21) cancer cells, treated with HS-OL (500 µg/ml or 350 µg/ml for breast cancer cells) for up to 72 hours. Data are mean of two cell lines for each group, with each cell line analyzed across three biological experiments. **b)** Cell viability analysis of colorectal (CS-CRC#2, CS-CRC#9), thyroid (Cal62, 8505c), and breast (MDA-MB-231, BCSphCs#21) cancer cells, treated with HS-CYN (500 µg/ml or 350 µg/ml for breast cancer cells) for up to 72 hours. Data are mean of two cell lines for each group, with each cell line analyzed across three biological experiments. **c)** Cell cycle analysis of colorectal (CS-CRC#2, CS-CRC#9), thyroid (Cal62, 8505c), and breast (MDA-MB-231, BCSphCs#21) cancer cells treated with HS-OL and HS-CYN for 48 hours. Data show the percentage of cells in subG0 G0–G1, S, and G2–M phases. **d)** Flow cytometry analysis of Caspase-3 activity in CR-CSC#2, Cal62, and B-CSphC#21 treated with HS-OL and HS-CYN for up to 48 hours. **e)** Analysis of Caspase-3 activity in colorectal (CS-CRC#2, CS-CRC#9), thyroid (Cal62, 8505c), and breast (BCSphCs#21, MDA-MB-231) cancer cells treated as in d. Data are mean of two cell lines for each group, with each cell line analyzed across three biological experiments.

Figure 3: HS-OL and HS-CYN activate DNA damage machinery in colon, thyroid and breast cancer cells. a) Heatmap showing the expression of DNA damage-related genes involved in ATM/ATR regulation of the G2-M checkpoint (2-ΔΔCt expression values) in CR-CSC#2, Cal62 and BCSphCs#21 cells after treatment with HS-OL and HS-CYN (500 for µg/ml) for 48 hours. **b)** *(upper panel)* Venn diagrams of common and exclusive upregulated genes with logFC > 1.5 in colorectal, thyroid and breast cancer cells treated with HS-OL. *(middle panel)* Venn diagrams of common and exclusive upregulated genes with logFC > 1.5 in colorectal, thyroid and breast cancer cells treated with HS-CYN. *(lower panel)* Venn diagrams of common and exclusive upregulated genes with logFC > 1.5 in colorectal, thyroid and breast cancer cells with both HSs. **c)** *(upper panel)* Immunoblot analysis of γ-H2AX in CR-CSC#2 Cal62 and BCSphCs#21 cells treated as in a. β-actin was used as a loading control. *(lower panel)* Optical density ratio of γ-H2AX in CR-CSC#2 Cal62 and BCSphCs#21 cells treated as in a. **d)** *(upper panel)* Immunofluorescence analysis of γ-H2AX (Red) in CR-CSC#2 Cal62 and BCSphCs#21 cells treated as in a. Nuclei were counterstained with Hoechest. Scale bar, 100 µm. *(lower panel)* Quantification of γ-H2AX signal normalized on Hoechst (MFI). Data are mean ± standard deviation of three independent experiments.

Figure 4: Combination of HS-OL and HS-CYN with radio or chemotherapy hampers the proliferation of colon, thyroid and breast cancer cells. **a)** *(left panel)* Cell viability analysis of CR-CSCs treated with of HS-OL or HS-CYN (500 µg/mL) and FOLFOX or FOLFIRI (1.25 µM) alone or in combination for 48 hs. *(middle panel)* Cell viability analysis of ATC cells irradiated with (Gy 5) or not treated with HS-OL or HS-CYN (500 µg/mL) alone or in combination with doxorubicin (200 nM) for 48 hs. *(right panel)* Cell viability analysis of breast cancer cells treated with HS-OL or HS-CYN (350 µg/mL) and doxorubicin (400 nM for BCSphCs#21 and 50 nm for MDA-MB231) alone or in combination for 48 hs. Data are expressed as a fold change over vehicle ± standard deviations of three independent experiments. **b)** Synergic diagram representing the combination index (CI), according to Chou-Talalay method, and computed by CompuSyn derived from cell proliferation described in a.

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F.O. executed the bioinformatics analysis; M.L.I., M.V.V., C.M. and G.S. wrote the manuscript. All authors revised the manuscript.

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Competing interests

The authors declare no competing interests.

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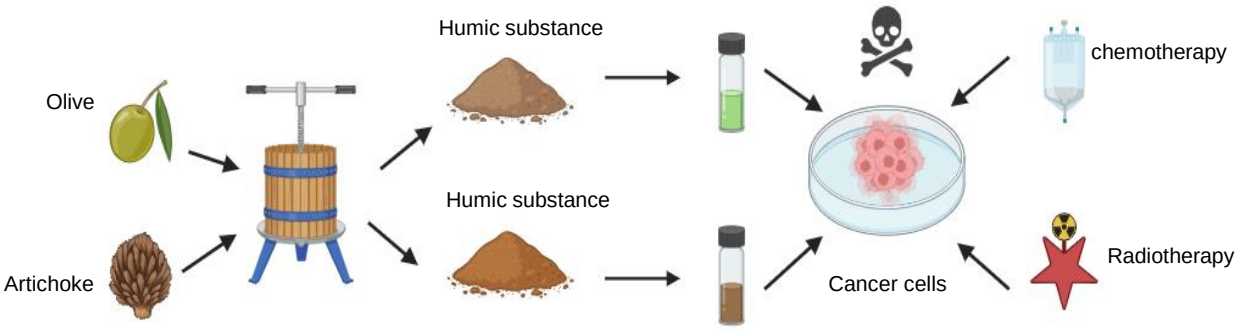
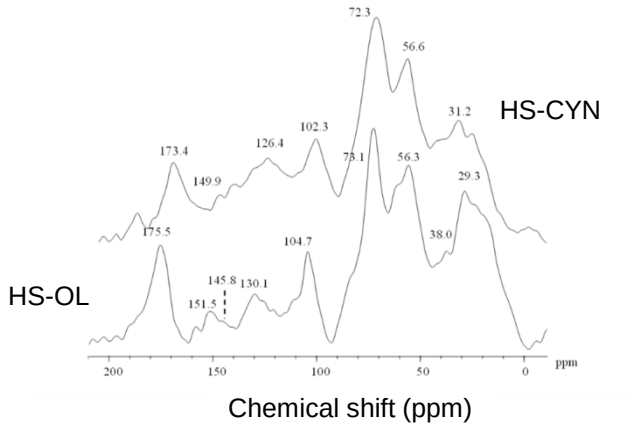


Graphical Abstract. humic substances were firstly derived from the decomposition of olive and artichoke (HS-OL and HS-CYN) and then solubilized in buffer saline solution. Colon, thyroid and breast cancer cells were exposed to extracts, either singularly or in combination with radio-chemotherapy.

a)

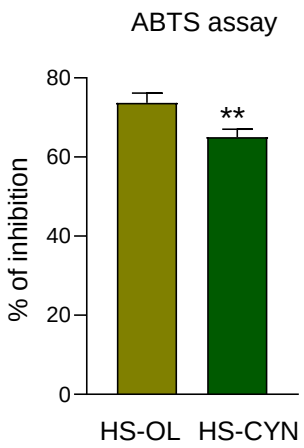


Chemical shift (ppm)

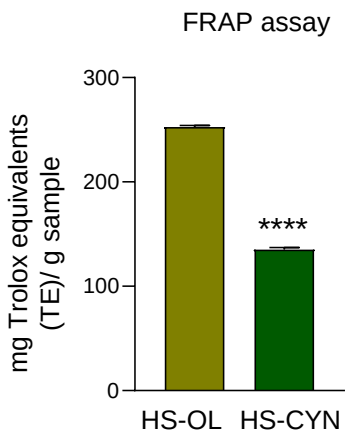
b)

	Carboxyl-C	O-Arylic-C	Aromatic-C	O-Alkyl-C	CH3O/CN	Alkyl-C	HB	A/AO	AR	LigR
Organic product	160-200 ppm	145-160 ppm	110-145 ppm	60-110 ppm	45-60 ppm	0-45 ppm				
HS-OL	11.2	6.5	14.6	25	20.1	22	0.8	0.9	0.5	3.1
HS-CYN	10.9	5.3	18.7	35.8	14.9	14.3	0.7	0.4	0.5	2.8

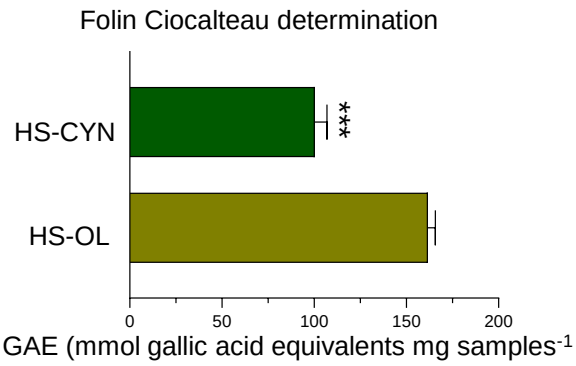
c)



d)



e)



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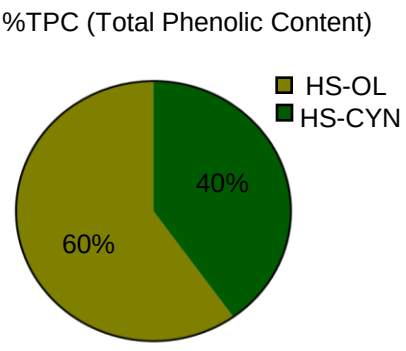
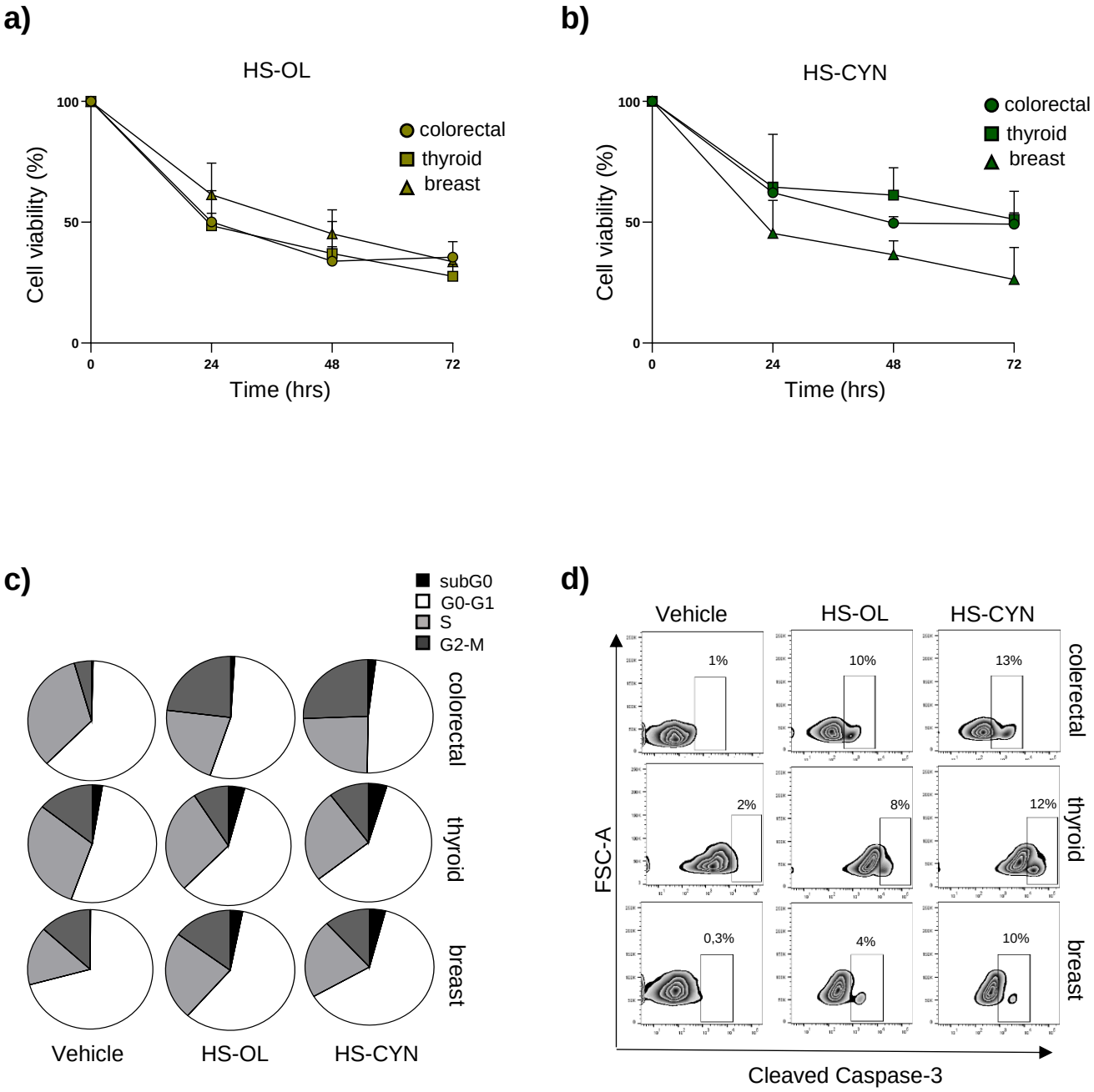


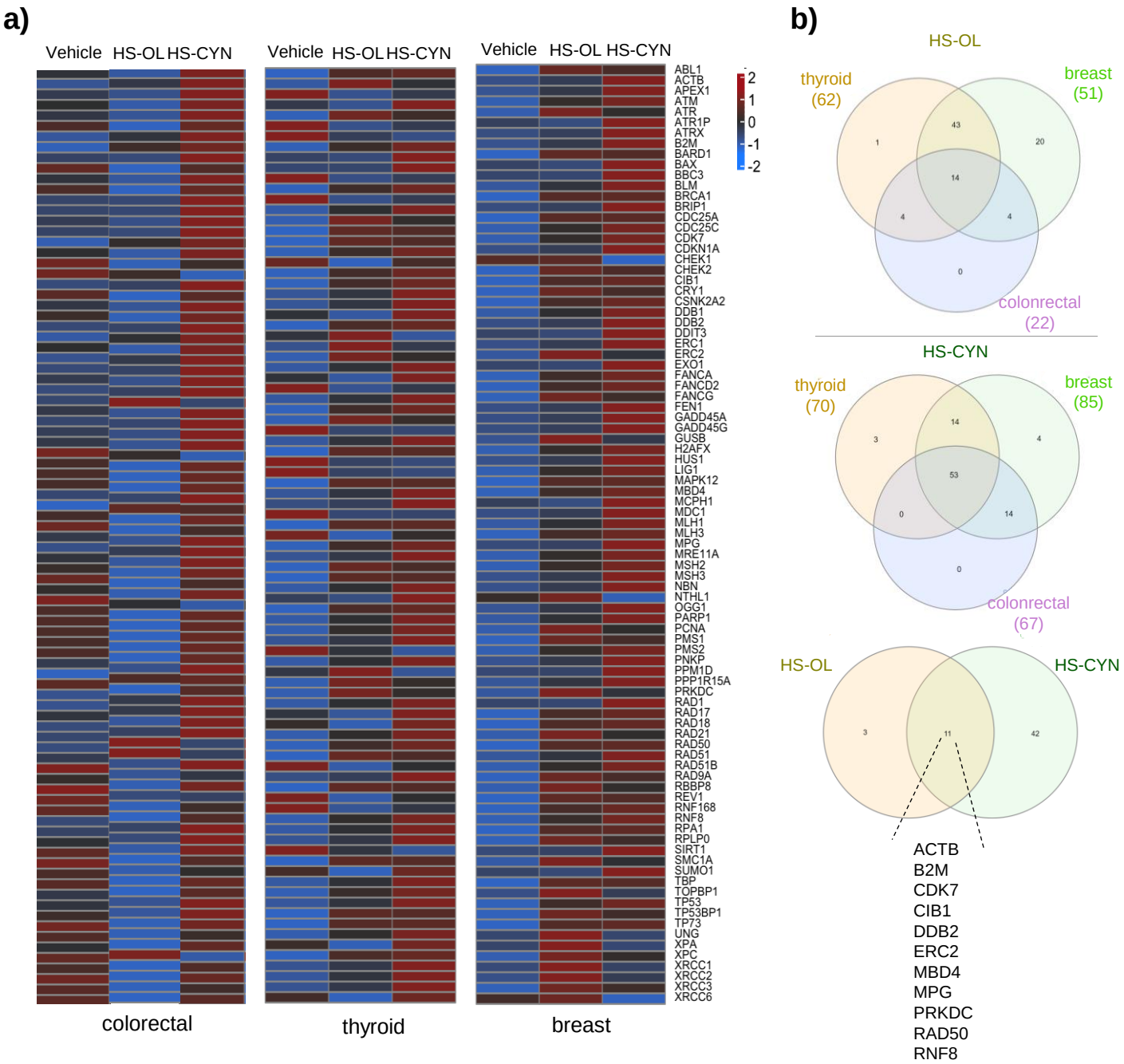
g)

Diameter(mm)	S.epidermis	L.monocitogenes	H.pylori	S.thyphi
HS-CYN	6.3±0.03b	7.1±0.06b	7.5±0.01b	7.9±0.04b
HS-OL	9.4±0.05c	8.3±0.02c	8.1±0.05c	8.5±0.08c
BSA	n.i.	n.i.	n.i.	n.i.
AMP+ Clavulanic acid	5.1±0.01a	4.2±0.04a	3.5±0.01a	4.8±0.02a

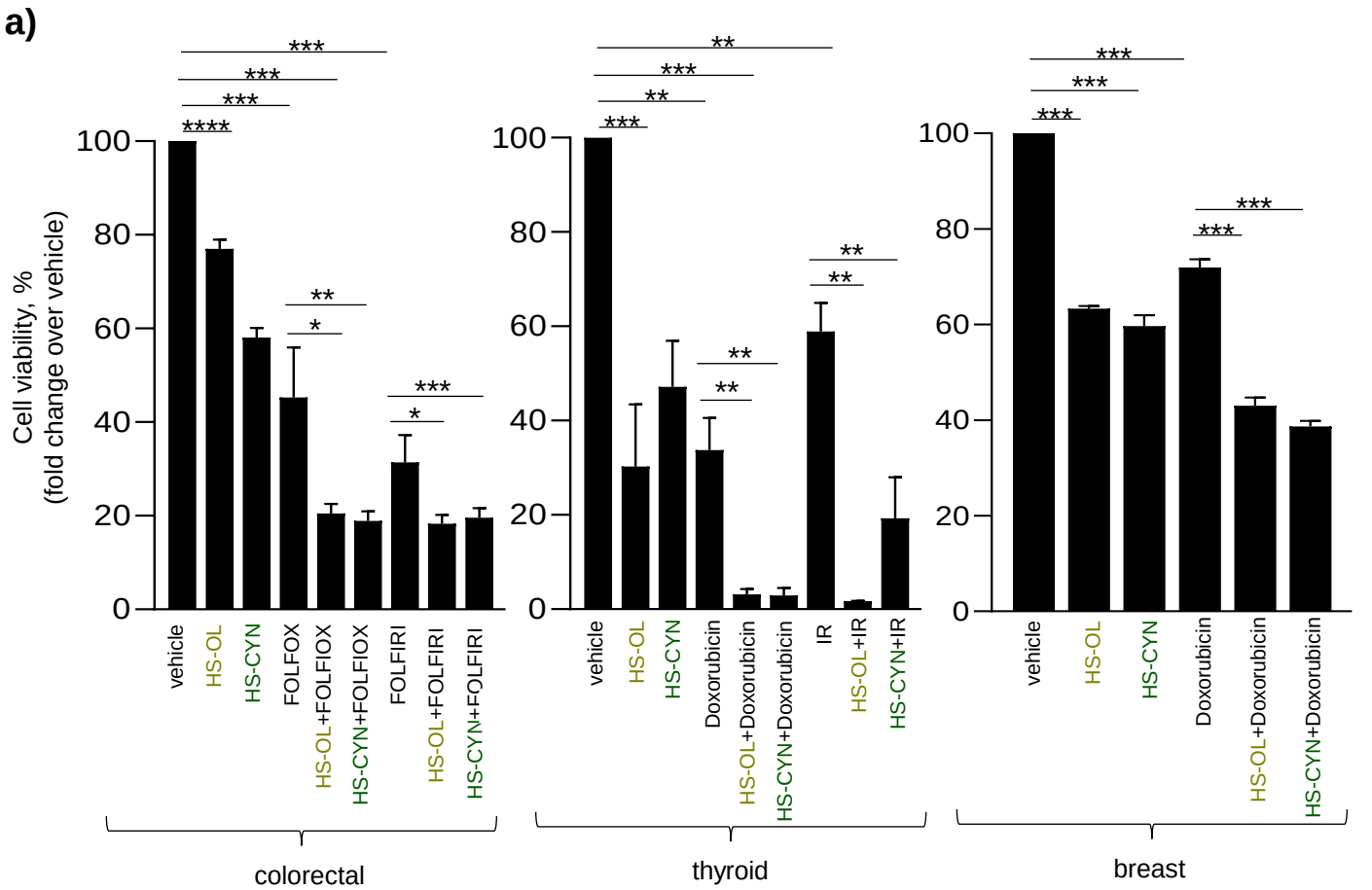
Lo Iacono M., Bianca P., Verrillo M.V., *et al.* Figure 1

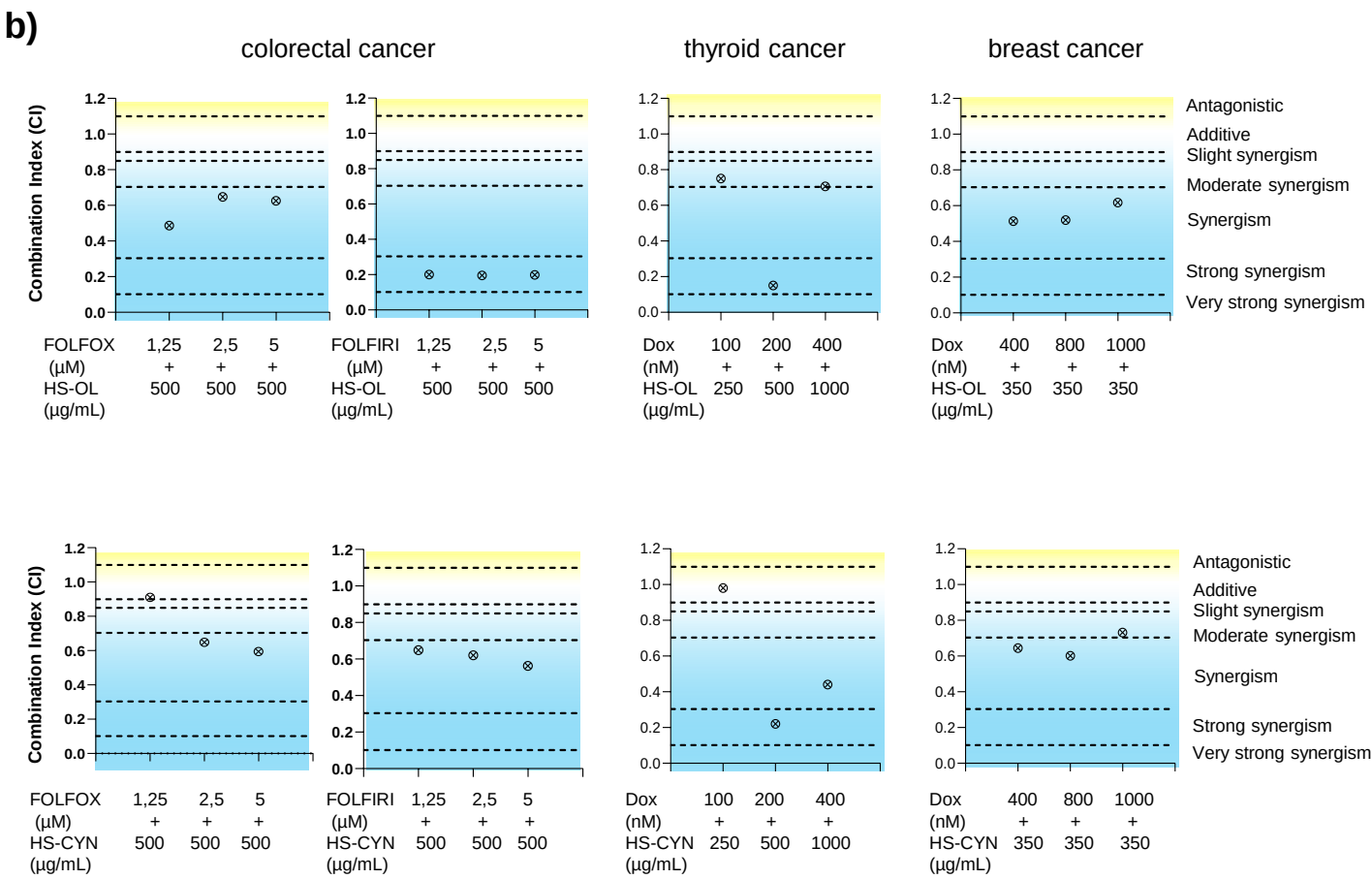


Lo Iacono M., Bianca P., Verrillo M.V., *et al.* Figure 2

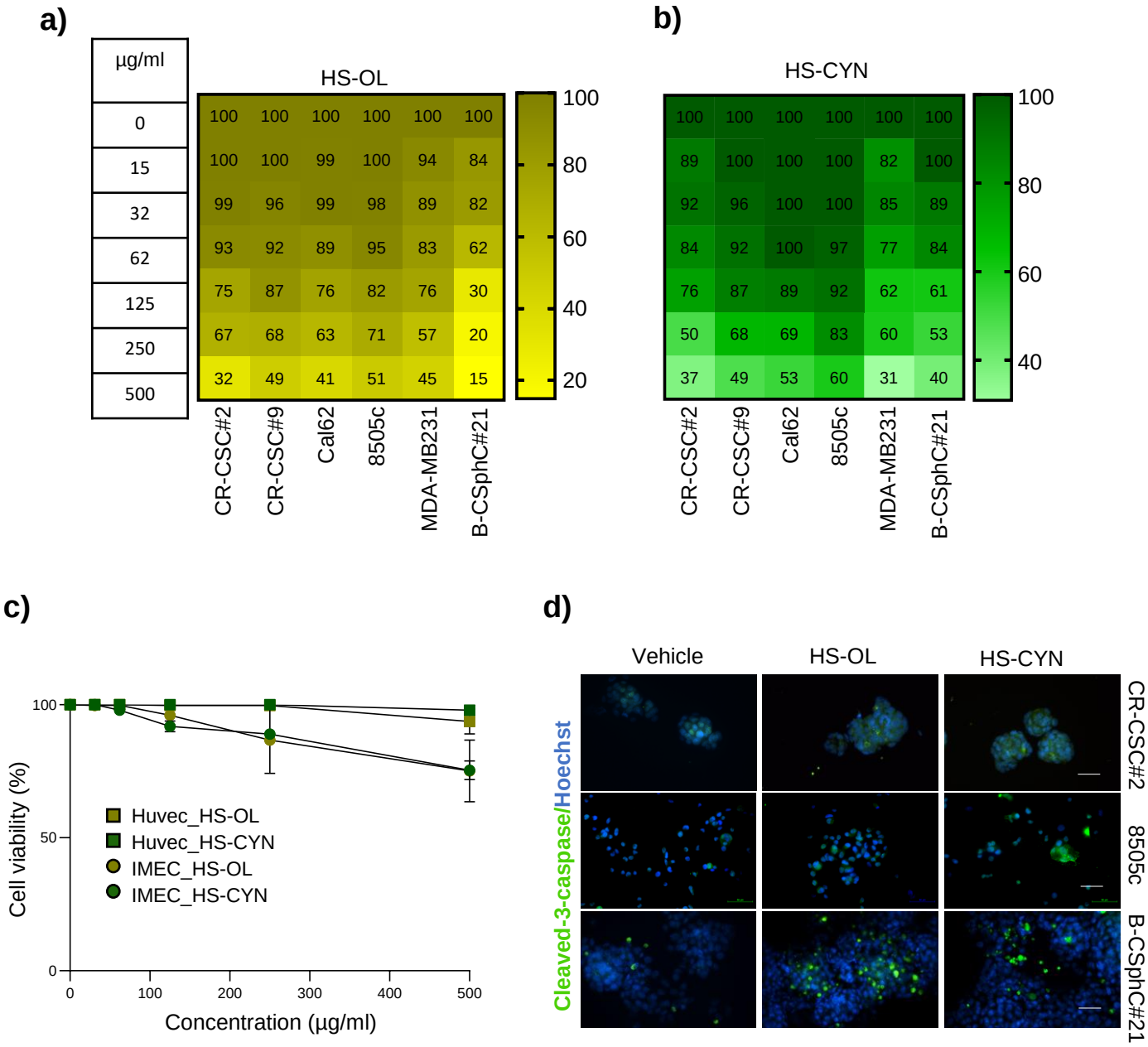


Lo Iacono M., Bianca P., Verrillo M.V., *et al.* Figure 3

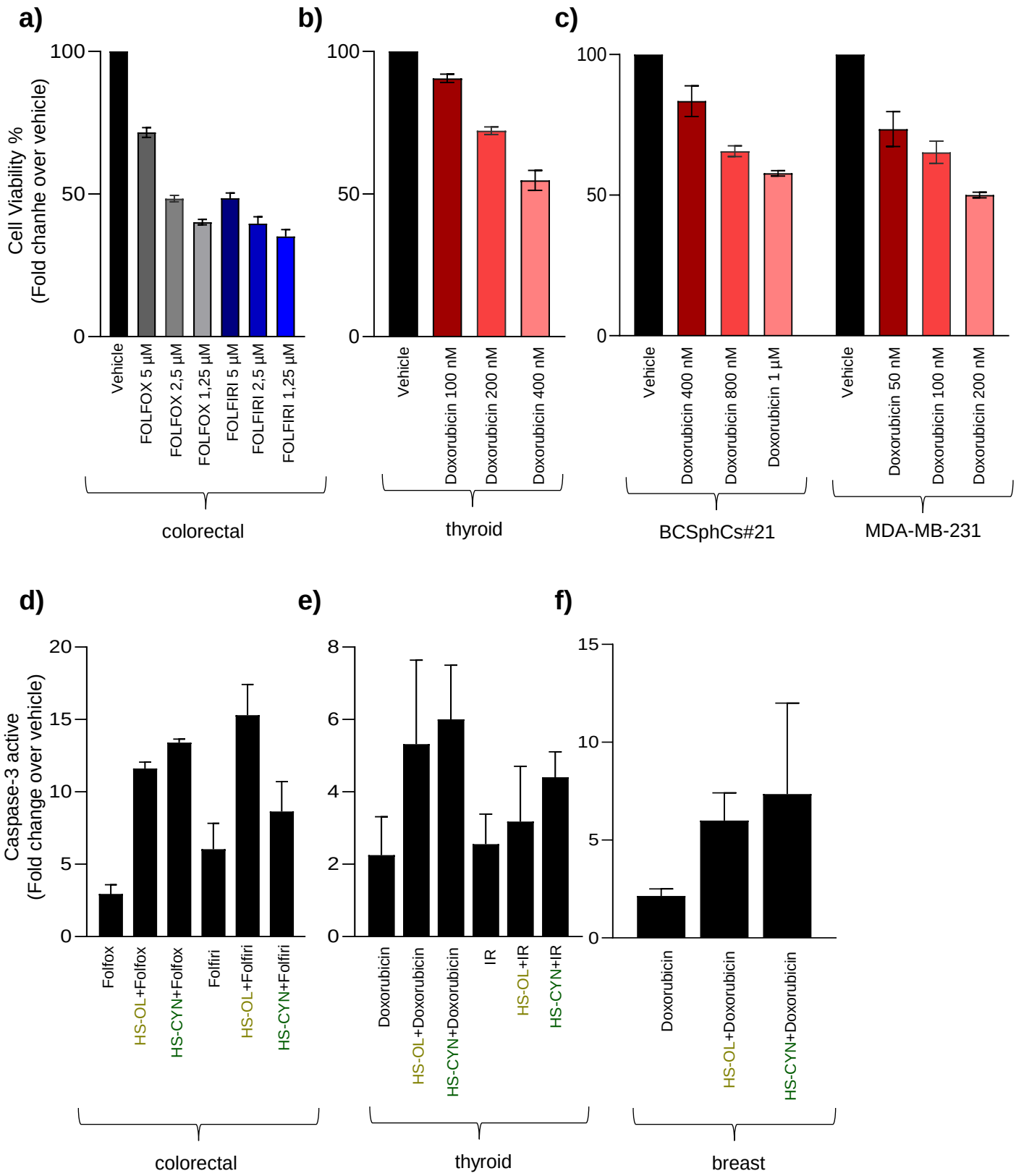




Lo Iacono M., Bianca P., Verrillo M.V., *et al.* Figure 4



Supplementary Figure 1. HS-OL and HS-CYN hamper cell viability in cancer cells. a) Cell viability analysis of colorectal (CS-CRC#2, CS-CRC#9), thyroid (Cal62, 8505c), and breast (MDA-MB-231, BCSphCs#21) cancer cells treated for 48 hours at the indicated concentration of HS-OL. **b)** Cell viability analysis of colorectal (CS-CRC#2, CS-CRC#9), thyroid (Cal62, 8505c), and breast (MDA-MB-231, BCSphCs#21) cancer cells treated for 48 hours at the indicated concentration of HS-CYN. **c)** Cell viability analysis of HUVEC and IMEC cells treated for 48 hours at the indicated concentration of HS-OL and HS-CYN. **d)** Immunofluorescence analysis of Cleaved-3-caspase in CS-CRC#2, 8505c and BCSphCs#21 cells treated with HS-OL and HS-CYN (500 µg/ml or 350 µg/ml for breast cancer cells) for up to 72 hours.



Supplementary Figure 2. HS-OL and HS-CYN in combination with radio-chemotherapy induce apoptosis in cancer cells. a) Cell viability of colorectal cancer cells (CS-CRC#2, CS-CRC#9) treated for 72 hours with the indicated concentration of FOLFOX and FOLFIRI. Data are mean ± standard deviations of two independent experiments for each cell line. **b)** Cell viability of thyroid cancer cells (Cal62, 8505c) treated for 72 hours with the indicated concentration Doxorubicin. Data are mean ± standard deviations of two independent experiments for each cell line. **c)** Cell viability of breast cancer cells (MDA-MB-231, BCSphCs#21) treated for 72 hours with the indicated concentration Doxorubicin. Data are mean ± standard deviations of two independent experiments for each cell line. **d)** Analysis of Cleaved-3-caspase in colorectal cancer cells (CS-CRC#2, CS-CRC#9) treated with HS-OL and HS-CYN (500 µg/ml) in combination with FOLFOX and FOLFIRI (1,25 µM) for up to 72 hours. Data are mean ± standard deviations of two independent experiments for each cell line. **e)** Analysis of Cleaved-3-caspase in thyroid cancer cells (Cal62, 8505c) treated with HS-OL and HS-CYN (500 µg/ml) in combination with Doxorubicin (200 nM) for up to 72 hours and irradiation (5 Gy). Data are mean ± standard deviations of two independent experiments for each cell line. **f)** Analysis of Cleaved-3-caspase in of breast cancer cells (MDA-MB-231, BCSphCs#21) treated with HS-OL and HS-CYN (350 µg/ml) in combination with Doxorubicin (100 nM) for up to 72 hours. Data are mean ± standard deviations of two independent experiments for each cell line.

Chapter 3

Combinatorial therapy targeting CD44V6-partners as novel strategy to kill colorectal cancer stem cells

CHAPTER 3 - COMBINATORIAL THERAPY TARGETING CD44V6-PARTNERS AS NOVEL STRATEGY TO KILL COLORECTAL CANCER STEM CELLS

ABSTRACT

The dissemination of cancer cells through metastatic tumors remains a critical clinical challenge, severely compromising the efficacy of current cancer therapies. Moreover, chemoresistance represents a significant hurdle in the effective treatment of this disease. In response, emerging therapeutic strategies have been explored, specifically targeting the molecular mechanisms that drive the reprogramming of cancer cells, leading to the acquisition of a more aggressive and treatment-resistant phenotype. This study explored the use of AMC303, a highly selective CD44V6 inhibitor, either alone or in combination with targeted therapies, within the context of the tumor microenvironment. Growth factors such as hepatocyte growth factor, present in the tumor microenvironment, are known to drive the reprogramming of cancer stem cells. Our results using colorectal cancer stem cells revealed that hepatocyte growth factor can trigger reprogramming even in the presence of AMC303. In fact, AMC303 is able to partially rescue this phenomenon. Notably, the inclusion of a PI3K inhibitor, Apitolisib was sufficient to reverse this effect, highlighting a promising approach for mitigating cancer stem cell reprogramming. As a result, combinatorial therapy presents a promising and effective approach for treating colorectal cancer.

INTRODUCTION

Colorectal Causes and statistics

Colorectal cancer (CRC) has a global prevalence rate of the third most common cancer diagnosed on earth and it is also the second cancer that causes death [1]. In 2020, CRC saw around 1.9 million newly diagnosed cases and scored 930,000 deaths across 25 countries. The highest rates were found in Australia, New Zealand, and the European regions, while the lowest rates were recorded in Africa and South Asia. As for the mortality rates, they followed a similar pattern, where Eastern Europe had the highest rates and Southern Asia the lowest. Nevertheless, the global burden of CRC is set to increase in the near future, with the World Health Organization projecting 3.2 million new cases and 1.6 million deaths by 2040. The prevalence rate of these cases will be the highest in countries whose Human Development Index will be high or very high [2]. CRC constitutes a prominent heterogeneous condition which has a remarkable contribution from the environmental aspects and genetic predispositions. The majority of CRC cases, which are sporadic, have no clear family history. Only about 5% of the cases are linked to inherited syndromes. The remaining 25% are considered familial CRC, where a hereditary component is evident but not fully understood or defined[3].

The journey of CRC goes through three stages; initiation, promotion, and progression. In the initiation phase, irreversible genetic damage occurs and this genetic damage pushes the epithelial cells in the intestinal lining to undergo further neoplastic changes. The majority of neoplastic lesions in the colon, such as adenomas and serrated polyps, demonstrate a loss of genomic and/or epigenetic stability at the very early stage. It is through the molecular and pathological event of cancer that the irreplaceably start and maintain the initiation and formation of CRC [4-6] Colorectal cancers are mainly firstly started as polyps that are abnormal crypts. These crypts are the precursors of early adenomas after which the advanced adenomas evolve [7].

One of the risk factors that is generally accepted for colorectal CRC is having a positive family history of the disease [8]. Besides, the other significant risk is excess body weight, which is linked to the higher chance of being diagnosed with the disease [9].

The relationship between diet and CRC has been thoroughly researched. Among the dietary factors linked to an increased risk of CRC are the consumption of processed and red meats [10-12], and sugar sweetened beverages [13], these are believed to influence gut immune response and

inflammation [14]. Finally, patients with inflammatory bowel disease (IBD) are also known to be at increased risk for CRC, due to long-term inflammation [15].

Colorectal cancer subtypes

The CRC Subtyping Consortium in 2015 identified four distinct molecular subtypes of CRC (CMS), each with unique characteristics [16]:

- CMS1 (immune): This subtype is marked by microsatellite instability (MSI) and features a high rate of mutations, particularly in CIMP and BRAF genes, while showing a low prevalence of somatic copy number alterations It is linked with significant lymphocyte infiltration and immune system activation. Additionally, it exhibits hypermethylation and reduced activity in the WNT signaling pathway [17].
- CMS2 (canonical): Distinguished by its epithelial properties, this subtype is defined by substantial chromosomal instability and a high count of SCNAs. It is associated with mutations in WNT and MYC genes, resulting in elevated activity of these signaling pathways [18].
- CMS3 (metabolic): This subtype displays a unique combination of global genomic and epigenomic features. It is characterized by mixed traits and involves metabolic reprogramming with disrupted pathways. There is increased activity in glutaminolysis and lipidogenesis within CMS3 [19].
- CMS4 (mesenchymal/ stromal): Known for its positive regulation of genes and overexpression of proteins associated with stromal infiltration, mesenchymal activation, and extracellular matrix interaction [20].

Colorectal Cancer Treatments and Challenges Due to Drug Resistance

Various treatment options for CRC include surgery, chemotherapy, radiation therapy, immunotherapy, and nutritional supplement therapy. However, these treatments often face challenges, including side effects and the potential for cancer cells to develop resistance, thereby reducing treatment effectiveness. Furthermore, the presence of temporal molecular heterogeneity—different cancer cell populations with distinct molecular profiles at different times—can affect treatment response and prognosis. Current treatment strategies for CRC, aside from the surgical removal of the primary tumor, heavily rely on chemo-cytotoxic agents and radiation therapy. These treatments, which include drugs like fluoropyrimidines, irinotecan, and oxaliplatin, work by disrupting the cellular replication process, thereby targeting rapidly dividing cells [21, 22]. In recent advancements, chemotherapy drugs such as oxaliplatin, irinotecan, and capecitabine have been developed. For advanced stages of CRC, the standard treatment typically combines 5-fluorouracil (5-FU) and leucovorin with either oxaliplatin or irinotecan [23]. Significant progress has also been made in CRC treatment with the introduction of monoclonal antibodies, such as Bevacizumab and Cetuximab. Although these targeted therapies have improved response rates, the five-year survival rate for metastatic CRC remains low, hovering just above 12 percent [24]. Additionally, antiangiogenic agents, which inhibit the formation of new blood vessels necessary for tumor growth, are employed to manage and control cancer progression [25]. For first-line chemotherapy in CRC, a combination of 5-FU, leucovorin, and irinotecan (known as the FOLFIRI regimen) is often recommended. Capecitabine, an oral chemotherapy drug, offers a convenient alternative to intravenous 5-FU for those who can tolerate it. Platinum-based drugs like oxaliplatin and cisplatin are also frequently used [26, 27]. In the FOLFOX regimen, oxaliplatin is paired with leucovorin and 5-FU. Furthermore, targeted therapies involving monoclonal antibodies, which block epidermal

growth factor (EGF) (e.g., cetuximab, panitumumab) or vascular endothelial growth factor (VEGF) (e.g., bevacizumab) signaling, have shown promising results when used alongside standard chemoradiotherapy [28]. Despite the efficacy of these conventional treatments, they can cause a range of adverse side effects, including fatigue, headache, muscle and stomach pain, diarrhea, and other gastrointestinal (GI) discomforts, which can be intolerable for patients and may lead to nonadherence with the treatment.

A significant challenge in treating CRC is the emergence of drug resistance. Approximately 50% of CRC patients exhibit resistance to 5-FU-based chemotherapies, making treatment less effective [29]. Additionally, colon cancer often shows resistance to ionizing radiation (IR), necessitating higher radiation doses for effective clinical treatment. This increase in dosage can lead to collateral damage to nearby healthy tissues and organs [30].

Challenges in Conventional Cancer Therapy

Conventional cancer therapies, including chemotherapy and radiation, often do not differentiate between normal and cancerous cells. In contrast, targeted therapies that inhibit specific pathways critical to cancer cell survival, such as epidermal growth factor receptor (EGFR), BRAF, c-MET, or HER2, are more specific and potentially less toxic. However, the heterogeneity of tumors, characterized by genetic, epigenetic, and metabolic diversity, complicates diagnosis and treatment. Factors in the tumor microenvironment, such as hepatocyte growth factor (HGF), support this heterogeneity by creating niches favorable for CSCs [31, 32]

Natural products

Innovative and promising cancer treatments have been investigated in natural compounds and their structural analogs, revealing significant chemical diversity. Furthermore, the unique molecular properties of these natural products contribute to their enhanced safety and efficacy [33]. Over the past few decades, the chemotherapeutic properties of natural compounds have significantly contributed to the development of various natural anticancer products for cancer prevention and treatment. Examples include paclitaxel, camptothecin, and irinotecan derived from plants, as well as doxorubicin sourced from bacteria [34]. The ability of dietary natural products to protect against the malignant transformation of cells is backed by extensive epidemiological evidence demonstrating a strong link between plant-based diets and a lower risk of cancer [35]. Curcumin, a polyphenolic compound extracted from turmeric, exhibits strong anti-cancer properties by influencing critical signaling pathways related to inflammation and cell growth, such as NF-κB and JAK2/STAT3, across various cancertypes, including breast and prostate cancers. In a similar vein, epigallocatechin gallate (EGCG), a key component of green tea, demonstrates significant antioxidant effects and has been shown to promote apoptosis while suppressing the growth of cancer cells by modulating pathways like retinoic acid receptor β (RARβ) and CDH1 (e-cadherin) [36]. The role of natural products in cancer prevention and metastasis is increasingly recognized, particularly in relation to CSCs, which are pivotal in tumor initiation, progression, and recurrence. Natural compounds have been shown to exhibit anti-cancer properties by targeting CSCs. These compounds can modulate signaling pathways associated with CSC self-renewal and differentiation [37]. For instance, some phytochemicals are able to reduce the expression of stemness markers like CD44 and CD133, which are commonly associated with CSCs [38].

Cancer stem cells

The concept of cancer-initiating cells, or CSCs, which were initially discovered in hematologic cancers, has also been identified in solid tumors like CRC. The hypothesis that these CSCs drive

tumorigenesis in colon cancer raises concerns about whether current treatments adequately target these tumorigenic cells, which are crucial for tumor growth and persistence. Research suggests that factors produced by the tumor microenvironment, such as EGF, insulin-like growth factor-1 receptor (IGF-IR), fibroblast growth factor-2 (FGF-2), VEGF, and cytokines like TGF-β, TNF-α, and IL-6, can revert differentiated cells to a more stem cell-like state [39].

Among the key properties of CSCs we found:

1. Self-Renewal: CSCs possess the ability to generate new stem cells that retain their full potential for proliferation and differentiation. This self-renewal is sustained by mechanisms involving proto-oncogenic pathways like Wnt/β-catenin and Notch [40]. Signals such as the Wnt pathway and the inhibition of β-catenin-dependent transcription play crucial roles in maintaining the self-renewal capacity of colon CSCs. HGF also helps preserve CSCs in their undifferentiated state [41].
2. Differentiation: CSCs can differentiate into a diverse progeny of cells, following a hierarchical process that replenishes tissues with mature, short-lived cells [42].
3. Homeostatic Control: CSCs can modulate and balance their processes of differentiation and self-renewal, maintaining tissue homeostasis [43].
4. Genomic Instability: During cancer progression, CSCs acquire more malignant traits through three main pathways of genomic instability: chromosomal instability, microsatellite instability, and the CpG island methylator phenotype [44].

Increasing evidence supports the view that many human cancers, including CRC, can be considered diseases driven by CSCs. The cancer stem cell model posits that malignancies arise from a small subset of cancer cells capable of self-renewal and pluripotency, which are critical for initiating and sustaining tumor growth [45]. Although CSCs make up a minor fraction of the total cancer cell population in CRC, they play a pivotal role in tumor initiation and maintenance. Hence, targeting CSCs presents a promising strategy for novel cancer treatments [46]. Compared to normal stem cells, CSCs are characterized by an unchecked capacity for proliferation. However, their slow cell cycling contributes to their resistance to chemotherapy and radiotherapy, as well as to tumor recurrence [47, 48].
CSCs are notably resistant to standard treatments, often leading to cancer recurrence after initial therapy. These cells can also migrate to other parts of the body, initiating new tumors in a process known as metastasis [49, 50]. In colorectal CRC, CSCs are identified by their high Wnt signaling activity and are typically found in a specific myofibroblast niche within the tumor environment [51]. The expression of the Wnt target gene CD44 further delineates the CSC population in both primary and metastatic CRC [51, 52]. CSCs resist drug treatments primarily through the activation of pro-survival signaling pathways. CD44, a receptor for hyaluronan (HA), is a key marker for CSCs across various cancers. When CD44 binds to HA, it can interact with EGFR, promoting survival and proliferation signals [53, 54].

CD44 role in cancer

CD44 is a complex transmembrane glycoprotein that binds to hyaluronic acid (HA), a critical component of the extracellular matrix (ECM) [55]. This protein is expressed in a range of human cell types, including embryonic stem cells, differentiated cells, and cancer cells. Alternative splicing during transcription produces two major isoforms: the standard form (CD44s) and variant forms (CD44v) [56, 57]. CD44 plays diverse physiological roles, including organ development, immune functions, and hematopoiesis. It is involved in processes like T cell differentiation, branching morphogenesis, cell proliferation, adhesion, and migration [58]. As a surface marker of CSCs, CD44 is crucial for tumor initiation and progression, and its aberrant expression contributes to the

formation of various cancers [57, 59-61]. CD44 is also significant in cancer invasion and metastasis across different tumor types [62]. Structurally, CD44 comprises three regions: the ectodomain, the transmembrane region, and the intracellular tail [56, 63]. The CD44v isoforms include an additional stem membrane-proximal segment, which can consist of single or multiple variant exons. These isoforms undergo further modifications through glycosylation and the addition of heparan sulfate or chondroitin sulfate [64, 65].

CD44 role in the maintenance of CSCs phenotype, therapy resistance and onset of metastatic disease

CD44 is implicated in driving CSC characteristics by activating pathways such as the platelet-derived growth factor receptor β and signal transducer and activator of transcription 3 (STAT3) signaling. The CD44s isoform, prevalent in breast CSCs, aligns with the gene profile of CSCs. Suppressing CD44s can disrupt CSC properties, while switching from CD44v to CD44s can alter CSC genotype and phenotype [66]. High CD44 expression is associated with a subgroup of invasive luminal breast tumor cells that exhibit a mesenchymal gene profile and play a crucial role in invasion [67]. CD44 plays a critical role in cancer progression and contributes significantly to resistance against anti-tumor therapies. Its aberrant expression is associated with lower sensitivity to treatments like the chemotherapy drug 5-FU. CD44-positive cells exhibit stem-like characteristics and overexpress genes linked to tumor stemness and chemoresistance [68]. Among the CD44 isoforms, CD44v6, which lacks heparin or heparan sulfate (HS) modifications, is particularly noteworthy [65]. This variant aids in chemotherapy resistance by helping cancer cells evade apoptosis and engage in autophagy, a process often linked to poor prognosis in CRC patients [69]. CD44v6 is crucial for the activation and signaling of c-MET and vascular endothelial growth factor receptor-2 (VEGFR-2) in various cancer cell lines and primary cells. Specifically, the extracellular domain of CD44v6 participates in receptor activation, while the intracellular domain is involved in downstream signaling [70]. This isoform plays a role in modulating the extracellular matrix, inhibiting tumor cell death, and enhancing cell movement, thereby promoting cancer progression [71]. Additionally, CD44v6 regulates c-MET internalization and is co-internalized with c-MET when induced by HGF [72]. c-MET, a receptor tyrosine kinase primarily found on epithelial cells, binds HGF, leading to c-MET dimerization, phosphorylation, and activation of various intracellular signaling pathways. This activation enhances cell proliferation, survival, and motility through pathways involving ERK1/2, PI3K/AKT, STAT3, and Rac1 [73, 74].

c-MET and HGF in tumor growth and metastasis

c-MET and HGF are essential for cell growth, migration, and epithelial cell survival [75]. Dysregulation of c-MET signaling, often due to c-MET gene mutations or autocrine HGF production, can drive tumorigenesis and metastasis [74]. Mechanistically, CD44v6's extracellular domain facilitates c-MET phosphorylation, while its intracellular tail supports downstream signaling and c-MET internalization upon HGF induction [76]. HGF's activation of c-MET induces colon cancer cell proliferation, motility, adhesion, and invasion, and contributes to the epithelial-mesenchymal transition (EMT) [77-80]The presence of CD44v6 is necessary for HGF-induced c-MET activation and subsequent ERK and STAT3 signaling, which promote cancer cell proliferation, survival, and migration. This activation also triggers pro-survival pathways and EMT, leading to resistance to anti-cancer therapies [81-84].

Tumor Microenvironment

The tumor microenvironment is a dynamic and complex entity, varying across different tumor types. It typically includes immune cells, stromal cells, blood vessels, and the extracellular matrix

(ECM). This environment is an active participant in cancer progression rather than a passive backdrop [85]. Components of the tumor microenvironment include:

- Immune Cells: Such as macrophages, T lymphocytes, tumor-associated dendritic cells (TADCs), eosinophils, granulocytes, natural killer (NK) cells, and B cells, which are involved in immune responses and can influence tumor dynamics.
- Stromal Cells and ECM: Stromal cells, like cancer-associated fibroblasts (CAFs), support tumor growth by secreting growth factors (e.g., transforming growth factor (TGF-β)), matrix-degrading enzymes (e.g., matrix metalloproteinases or MMPs), and angiogenic factors (e.g., VEGF) [86].

- Cytokines and Growth Factors: Produced within the tumor environment, these factors regulate the differentiation, activation, and function of various cells, contributing to the complex interactions that support cancer cell survival and proliferation.

The ECM also serves as a reservoir for cytokines and growth factors, such as VEGF, FGF, PDGF, and TGF-β, which are released by proteolytic enzymes like MMPs [85]. These factors are integral to processes such as cancer initiation, growth, invasion, angiogenesis, and metastasis. Understanding the roles of CD44, particularly CD44v6, and the tumor microenvironment in CRC offers insights into the development of resistance and metastasis, emphasizing the need for targeted therapies that address these specific mechanisms. Cytokines, secreted by both tumor and immune cells, play complex roles in cancer dynamics. They can either enhance tumor growth and survival or contribute to anti-tumor defenses. Chronic inflammation, driven by inflammatory mediators like tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-17, disrupts anti-tumor immunity and accelerates tumor progression [87, 88]. Certain cytokines, such as TGF-β, interferon IFN-γ, IL-2, and IL-15, are known to suppress tumors. The multifaceted actions of cytokines within the tumor microenvironment elucidate their dual role in either promoting or hindering tumor development. Cytokines and growth factors released by tumor cells can significantly alter the local tumor microenvironment. They modulate the immune response, reduce the expression of vascular cell adhesion proteins, and induce angiogenesis. Key players in these processes are the cytokines produced by activated innate immune cells, which stimulate tumor growth and progression. Furthermore, soluble factors from cancer cells can recruit and activate inflammatory cells, which in turn, further drive tumor progression [88].

Cancer-Associated Fibroblasts (CAFs)

Cancer-associated fibroblasts (CAFs) are instrumental in promoting EMT, cell scattering, and migration in a HGF-dependent manner. These fibroblasts, along with recombinant HGF, support cancer cell survival and contribute significantly to both primary and acquired resistance to targeted therapies, including those inhibiting the EGFR. Additionally, myofibroblasts enhance the cancer stem cell phenotype by promoting Wnt signaling through HGF production [51].

Molecular Signaling Pathways driving tumor progression

At the molecular level, the activation of receptor tyrosine kinases (RTKs) such as EGFR, VEGFR, FGFR, and PDGFR stimulates the MAPK and PI3K/Akt/mTOR pathways. These pathways are crucial for normal cell homeostasis. Receptor tyrosine kinases (RTKs) are pivotal in tumor growth and metastasis. They provide essential signals that drive cellular transformation, proliferation, and invasion [89]. Studies have highlighted the expression of several RTKs, such as the EGFR, c-MET, insulin-like growth factor receptor (IGFR), and VEGFR in malignant pleural mesothelioma (MPM) [90-94]The HGF/c-MET signaling pathway is notably associated with acquired resistance to EGFR

inhibitors in non-small cell lung cancers with EGFR mutations [95]. Therefore, it is critical to target c-MET alongside complementary targets to effectively kill cancer cells and prevent resistance. When treated with PI3K/AKT inhibitors, there is an observed upregulation or activation of RTKs like EGFR, HER3, and IGFR1. This response can limit the effectiveness of these therapies by either reactivating the PI3K pathway or triggering alternative compensatory pathways [96, 97]. This underscores the importance of combining PI3K inhibitors with RTK blockers to enhance therapeutic outcomes.

- The **MAPK pathway** primarily drives cell proliferation via the RAS/RAF/MEK/ERK cascade.
- The **PI3K/Akt/mTOR pathway** regulates various cellular functions, including proliferation, differentiation, metabolism, and survival.

Oncogenic mutations in genes like KRAS and BRAF, or the activation of WNT, MYC, and TGF-β signaling pathways, lead to the dysregulation of these processes. This results in uncontrolled cell proliferation, disrupted cell cycle regulation, and altered interactions between the immune system and stromal components [98].

PI3K/Akt/mTOR Pathway in Cancer

The PI3K/Akt/mTOR signaling pathway is crucial in various cancer-related processes. It regulates the cell cycle, contributes to tumor angiogenesis, influences invasion and metastasis, controls apoptosis and autophagy, and is involved in drug resistance [99]. Mutations in the PI3K gene are commonly found in colorectal cancer, and inhibitors targeting PI3K can arrest cell growth by halting the cell cycle in the G1 phase and inhibiting mTOR activity. Akt, a key component of this pathway, phosphorylates mTOR and other downstream targets to promote cell survival, inhibit apoptosis, and support the proliferation and survival of CRC. The PI3K pathway thus plays a significant role in colorectal cancer progression by enhancing cell proliferation, extending cell survival, and preventing apoptosis [100]. Researchers are making significant strides in developing PI3K inhibitors, which have shown promising results in treating various solid tumors, including breast, prostate, and colorectal cancers.

Targeted Therapies and Tyrosine Kinase Inhibitors (TKIs)

Targeted therapies, such as tyrosine kinase inhibitors (TKIs), have shown promise by selectively inhibiting oncogenically activated signaling pathways. These agents specifically block critical pathways that cancer cells rely on, potentially leading to more effective treatments with fewer side effects compared to conventional therapies [101].

AMC303

The peptidergic inhibitor AMC303, which allosterically binds to CD44v6, was investigated for its inhibitory effects on autophosphorylated c-MET activation, and its impact on tumor growth and metastases during a trial. The result showed that AMC303 inhibited c-MET, VEGFR2 and RON phosphorylation. Moreover, AMC303 inhibited c-MET activation in cells. AMC303 attenuated both primary and metastatic tumor growth by increasing apoptosis and reduction of angiogenesis.

Apitolisib (GDC-0980)

Apitolisib (GDC-0980) is a potent, orally available dual inhibitor of PI3K and mTOR. It effectively targets class I PI3K isoforms (PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ) with a high level of precision and

also inhibits mTOR kinase. By modifying the structure of pictilisib, researchers developed GDC-0980 to combine mTOR inhibition with PI3K inhibition [102]. In a Phase I clinical trial involving patients with advanced solid tumors, including colorectal cancer, Apitolisib demonstrated moderate but sustained anti-tumor activity at a daily oral dose of 30 mg, with a tolerable safety profile [103].

Combinatorial Therapies

Monotherapies often fall short in cancer treatment, sometimes leading to significant side effects or even fatal outcomes. Tumors consist of diverse cell types, and following successful treatment, some cells may remain and contribute to tumor recurrence. Prolonged exposure to radiation or chemotherapy can also lead to resistance, diminishing treatment efficacy. One promising strategy to address these challenges is combinational therapy, which integrates different treatment modalities to potentially complement each other, reduce side effects, and overcome resistance [104]. Combination therapies, which may include radioimmunotherapy, radiochemotherapy, or dual drug regimens, can mitigate complications associated with side effects and drug resistance due to their non-overlapping mechanisms of action [105, 106]. Targeting multiple nodes in oncogenic signaling pathways simultaneously is considered an effective approach to inhibit key cancer-promoting pathways [107]. By attacking cancer cells on multiple fronts, these combinations can counteract cellular defense mechanisms, inhibit redundant pathways, and tackle emerging resistance [108].

MATERIALS AND METHODS

Isolation and maintenance of CR-CSphCs

Colorectal cancer sphere cells (CR-CSphCs) were sourced from human tumor tissue samples in accordance with the Human Experimentation Policy of Policlinico “Paolo Giaccone”, Palermo (authorization CE9/2015), as previously detailed. Initially, CRC specimens were finely chopped using surgical scissors and then enzymatically digested with collagenase (0.6 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) and hyaluronidase (10 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) in DMEM medium, under continuous shaking (150 rpm) at 37 °C for 30 minutes. The resulting suspension was subsequently centrifuged at 200× g for 5 minutes, and the cell pellet was resuspended in serum-free stem cell medium (SCM) supplemented with recombinant human FGF-basic (100 ng/mL, Peprotech, Cranbury, NJ, USA) and recombinant human EGF (50 ng/mL, Peprotech, Cranbury, NJ, USA). The cells were then cultured in ultra-low attachment culture flasks at a density of 1 × 10^5 cells/mL. Passage of the cells was carried out by dissociating sphere cells using Accutase (ThermoFisher Scientific, Waltham, MA, USA) once they reached a diameter of 50–100 µm. All experiments utilized CR-CSphCs at early passages (<20). In order to demonstrate the efficacy of CD44v6 inhibition in targeting CRC stem cells, we took advantage of our broad collection of primary CRC sphere cells, fully characterized for their consensus molecular subtype (CMS), and mutational background (**Table 1**).

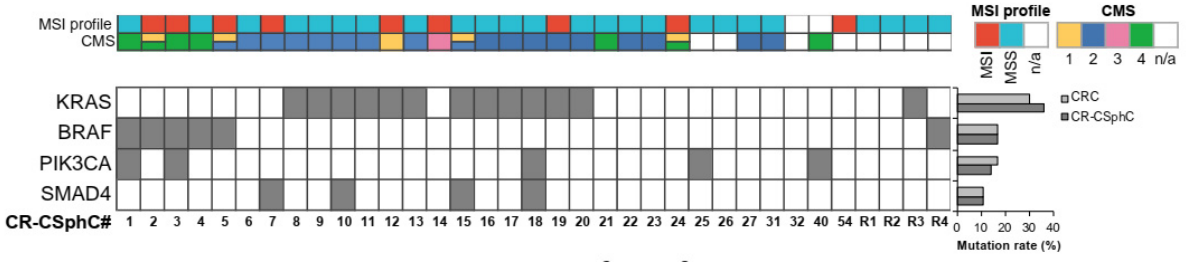


Table 1. Mutational, MSI and CMS profile of CR-CSphC.

CMS2 CRC cells underwent starvation of growth factors (EGF and bFGF) overnight in a serum-free Advanced DMEM/F12 medium (Gibco,USA), supplemented with 10 mM HEPES (EuroClone, Italy), 1 mM N-acetyl-l-cysteine (Sigma Aldrich; #A9165), 100x N2 supplement (Gibco, #17502001), 50X B-27 (Gibco), 10 mM Nicotinamide (Sigma Aldrich; #N0636), L-Glutamine (200Mm, EuroClone).

Treatments of CR-CSCs: vehicle, Human HGF (40 ng/ml), and (5 ng/ml) (Peprotech), AMC303 (1mM) and (10 μM) (Polypeptide group, Amcure), or HGF + AMC303 at lower (10 μM) or higher (1mM) concentration, Apitolisib (100 nM) . Before exposure to HGF, cells have been pre-treated with AMC303 or Apitolisib for 3 hours. Huvec cells were purchased by Lonza (C2519AS) and cultured according to the manufacturer's instructions. were seeded in Endothelial cell basal medium2 (Lonza), supplemented with EGM2 single Quots (Lonza). HUVEC cells after 24h of starvation were pretreated with AMC303, and after 3h were treated with VEGF (10 ng/ml) for 1h.

Cell proliferation assay

To evaluate cell proliferation, CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega) was used according to the manufacturer's instructions, and luminescence was measured by using Infinite F500 (Tecan). For cell titer glo , CRC cells were seeded in a U.L 96-well plate at a density of 3000 cells/well, and maintained under starvation conditions overnight. Subsequently, the cells were pretreated with AMC303 (10 μM) for 3 hours. Following this pretreatment, the cells were exposed to the HGF (40 ng/ml), cell titer glo was read over 72 h. Moreover 3000 HUVEC cells plated in 96 well plates and after 1 night starvation were treated with AMC303 for 3 h and then with VEGF (10 ng/ml) for 1 h. Then cell titer glo was read over 48 h. Additionally, we analyzed proliferation assay by row counting, to this aim 500,000 CRC cells were plated in U.L 6 well plate for each condition and after treatment we counted them in day 3,7, and 10, Following each counting procedure, we replaced the 500,000 cells.
To compare the effects of AMC303 and Apitolisib, row counting was performed for : Vehicle, HGF (5 ng/ml), HGF + AMC303 (10 μM), HGF+ Apitolisib, HGF + AMC303 (10 μM) + Apitolisib (100 nM) and Apitolisib alone. Before exposure to HGF, cells have been pre-treated with AMC303 and Apitolisib for 3 hours. on the days 3, 7, and 10 the cells were counted .
For Determination of inhibitors IC50 Values, five CRC cell lines were seeded in DMEM advanced medium within 96-well plates, at a density of 3000 cells per well. Each inhibitor was tested in triplicate at various concentrations: BAY 80-6946 (*copanlisib*) (Catalog No.S2802, Selleckchem) 10 nM, 50 nM, 100 nM, GDC-0980 (*Apitolisib*) (Catalog No.S2696 , Selleckchem) 50 nM, 100 nM, GDC-0068 (*Ipatasertib)* (Catalog No.S2808, Selleckchem) 200 nM, 1 μM, GSK1120212 (*Trametinib*) (Catalog No.S2673, Selleckchem) 100 nM, Crizotinib (Catalog No.S1068, Selleckchem) 10 nM, 30 nM, and C188-9 (Catalog No.S8605, Selleckchem) 10 nM. Cell viability was measured using the CellTiter-Glo assay over a 72-hour incubation period.

Clonogenic assay

To investigate the effect of AMC303 in the presence of HGF on the Clonogenic potential of CRC cells after 1 night starvation followed by a 3-hour pretreatment with AMC303 (10 μM). Subsequently, the cells were treated with HGF (5 ng/ ml), we plated 1, 2, 4, 8, 16, 32, 64, 128 cells per well in 96 well plates. Treatments with AMC303 were replenished daily, while HGF was administered every 48 hours. Groups: vehicle, HGF, HGF + AMC303. This experiment was evaluated with the Extreme Limiting Dilution Analysis (ELDA) 'limdil' function (**http://bioinf.wehi.edu.au/software/elda**). This experimental setting was performed also for : Vehicle, HGF (5 ng/ml), HGF + AMC303, HGF +AMC303 + Apitolisib (100 nM). Cells were pretreated by AMC303 and Apitolisib and after 3 h treated by HGF. Treatment by AMC303 was repeated every day, while for Apitolisib and HGF was twice a week.

Colony forming assay

In the next step, a colony-forming assay was conducted to investigate the potential role of AMC303 in inhibiting the stemness of CRC cells in the presence of HGF. The cells were starved overnight before being divided into different groups: vehicle, HGF, AMC303, HGF + AMC303. Cells pretreated with AMC303 (10 μM) and after 3h were treated with HGF (5 ng/ml). Treatments with AMC303 were replenished daily, while HGF was administered every 48 hours. For the colony-forming assay, 2×10^3 CR-CSphCs were seeded on low-melting agarose (Agarose Sea Plague Agar, # 50101, Lonza), and maintained for 21 days. Crystal violet staining (0.01 %) was used to reveal cell colonies and the clonogenic potential of CR sphere cells was assessed using ImageJ software (Bethesda, MD, USA). Additionally, this assay was conducted for other groups: vehicle, HGF, HGF + AMC303 (10 μM) , HGF + AMC303 (10 μM) + Apitolisib (100 nM), HGF + Apitolisb and Apitolisib alone.

Western blot

Cells underwent starvation overnight, then they have been treated with vehicle, human HGF (40 ng/ml), AMC303 (1 mM) and (10 μM), HGF + AMC303 at lower (10 μM) or higher (1 mM) concentration. Before exposure to HGF, cells have been pre-treated with AMC303 for 1 hour. Cells were collected one hour after HGF treatment and used to obtain protein extracts. We investigated the activation of HGF-driven pathways, including phosphorylation of c-Met, and AKT by western blot. For western blot the cells after treatment were collected and washed with PBS, before being lysed using a buffer containing 1% triton (Bio-Rad), 0.1 nM sodium orthovanadate (Sigma-Aldrich), supplemented with protease and phosphatase inhibitor cocktails, 10 mM sodium butyrate (Sigma-Aldrich) and 1 mM PMSF (Sigma-Aldrich).

Total protein extracts were loaded onto SDS-PAGE gel and blotted on nitrocellulose membranes. Following the incubation with blocking solution (5% non-fat dry milk, Santa Cruz Biotechnology, PBS 0.1% Tween 20), membranes were exposed overnight at 4 °C to primary antibody. phospho met (Tyr 1234/1235, RB, CST, 1:1000), Phospho AKT (Ser473, rabbit, , CST, 1:1000 dilution), AKT (9272 rabbit, CST, 1:1000 dilution). For protein expression , β-actin (8H10D10,mouse,CST,1:1000 dilution) was used as endogenous control. Huvec cells after one night of starvation (-VEGF), were treated by AMC303 (10 μM) for 3h, then VEGF (10 ng/ml) was added and after 1h protein extraction and western blot were conducted. Groups include: vehicle, VEGF, AMC303+VEGF. Primary antibodies: Phospho-VEGF Receptor 2 (Invitrogen, 1:1000), VEGF Receptor 2, β-actin (8H10D10,mouse, CST,1:1000 dilution) was used as endogenous control. Furthermore, We selected the highest non-toxic concentration for each inhibitor, and CRC cells after one night starvation, were treated by the inhibitors and after 3h HGF(5 ng/ml) was added.

After 30 min, total protein was extracted and western blot was performed. primary antibodies: Phospho Mek (SER 217-221,Rb, CST, 1;1000), Mek (9122S, Rb, 1:1000 dilution), Phospho AKT (Ser473, rabbit, , CST, 1:1000 dilution), AKT (9272 rabbit, CST, 1:1000 dilution), Phospho ERK(MAPK, P-P44/42, 9101S, CST, 1:1000 dilution), ERK (P44/42, MAPK, 157FS, CST, Rb, 1:1000 dilution), and β-actin (8H10D10, mouse, CST, 1:1000 dilution) was used as endogenous control. According to the result of western blot, the highest non-toxic concentration of Apitolisib was selected. To compare the effects of Apitolisib and AMC303 on the inhibition of c-Met and downstream pathways, CR-CRCs underwent starvation overnight and then pretreated by AMC303(10 μM) , and Apitolisib(100 nM) for 3h, then were treated by HGF (40 ng/ml), after 1h protein extracted for western blot primary antibodies: Phospho-Met, Met, Pi3kinase, STAT3, and β-actin. Protein levels of WNT/Beta catenin pathway for the cells treated with Vehicle, HGF (5 ng/ml), HGF + AMC303 (10 μM), or HGF + AMC303 (10 μM) + Apitolisib (100 nM), and HGF + AMC303 (10 μM) (pretreated by AMC303 and Apitolisib following treatment with HGF after 3h, after 3 days protein was extracted) western blot was conducted using Beta catenin (purified mouse anti beta catenin, MAB, BD Transduction Laboratories, mouse, 1:1000), Non- phospho Beta catenin (S33/S37/T41 mab, RB, CST, 1:1000), and β-actin (8H10D10, mouse, CST, 1:1000 dilution) was used as the endogenous control.

RNA isolation and gene expression analysis

To assess the differential effects of AMC303 both alone and in combination with Apitolisib, we conducted real-time PCR (qPCR) analysis. Our evaluation focused on the expression levels of key genes involved in Epithelial-mesenchymal transition, stemness, DNA damage repair, and chemoresistance. By comparing these expression profiles, we aimed to elucidate the molecular impacts of the AMC303 and its interaction with the inhibitor, providing insights into their potential therapeutic implications.

Total RNA was obtained (vehicle, HGF (5 ng/ml), HGF+AMC303(10 μM), HGF+Apitolisib (100 nM), Apitolisib alone, and HGF+AMC303+Apitolisib) using the TRIzol™ Reagent (Invitrogen), and 1 μg of total RNA, was retro-transcribed using the High-Capacity cDNA Reverse Transcription Kit (AppliedBiosystems). The expression analysis of genes involved in Epithelial-mesenchymal transition, stemness, DNA damage repair, and chemoresistance was evaluated using specific primers. To analyze the genetic variations, We conducted mRNA expression profiling using CARD-PCR using SBCA88741 Quantinova PCR Custom panels (384 wells), and Quantiniva syber green PCR kit (Qiagen) to evaluate the expression levels of genes associated with EMT, stemness, and chemoresistance

RESULTS

AMC303 is a novel CD44v6 inhibitor that has demonstrated potential in modulating colorectal cancer progression and metastasis. To investigate its effects, western blot analysis of CR-CSCs pretreated with two different concentrations of AMC303, in presence or absence of HGF, was performed. The result of this western blot showed the expression level of protein involved in MAPK, PI3K and JAK/STAT pathways. In fact, AMC303 can decrease the expression of phospho-met, but there is an over expression in the level of P-MEK, P-STAT3 and P-AKT in presence of

HGF. Thus, our results show that AMC303 is not able to impair the effect of HGF completely (**Figure 1**)

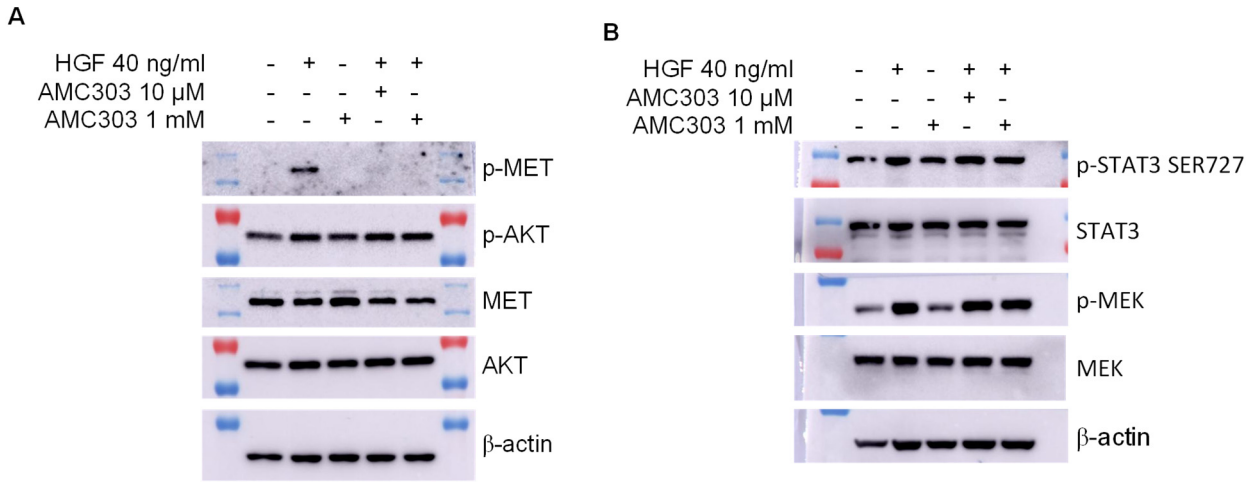


Figure 1. The expression of p-MET, MET, P-AKT, and AKT in (**A**) and P-STAT3(ser727), STAT3, P-MEK, and MEK (**B**) were analyzed by western blotting. Beta Actin was endogenous control.

AMC3 hamper CR-CSphCs and HUVEC cell viability

A cell proliferation assay on CRC cells treated with AMC303 and exposed by HGF was conducted up to 72 h. Our findings revealed that, in the presence of HGF, AMC303 significantly reduced the number of viable cancer stem cells. These results indicate that AMC303 may effectively inhibit the growth and dissemination of CRC (**Figure 2A-B**). AMC303 significantly reduced cell viability compared to HGF treatment alone. These results suggest that it effectively targets the HGF-MET signaling pathway, a critical survival mechanism for colon cancer stem cells. Additionally we examined the effects of AMC303 on HUVEC cell proliferation in presence of VEGF (**Figure 2C**). By targeting CD44v6, AMC303 is able to impede its binding to the coreceptor VEGFR2, leading to altered VEGFR signaling, in presence of the ligand VEGF. AMC303 might indirectly inhibit downstream targets of VEGF signaling, such as PI3K-AKT or MAPK pathways, leading to reduced cell survival and proliferation.

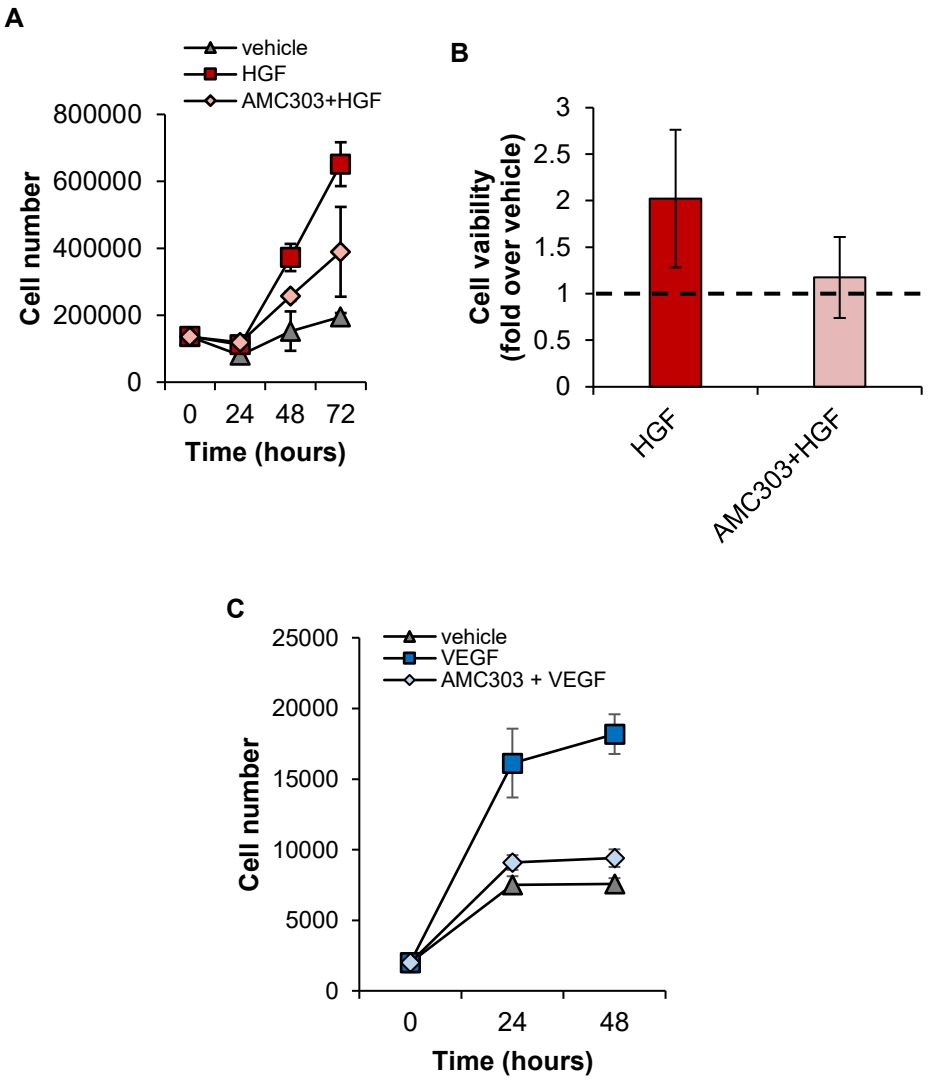


Figure 2. Quantitative analysis of cell proliferation of CR-CSphCs and HUVEC cells **A and B.** Cell viability of CRC cells following AMC303 treatment in presence of HGF, measured by the Cell Titer-Glo assay. Error bars represent standard deviation. **C.** Cell viability of HUVEC cells following AMC303 treatment in presence of HGF, measured by the CellTiter-Glo assay.

To determine the clonogenic potential of CRC cells in response to AMC303 in presence of HGF , a clonogenic assay was performed. Cells treated with AMC303 exhibited a significant reduction in clonogenic potential compared to HGF-treated cells (**Figure 3A**). These results suggest that AMC303 inhibits the proliferation and survival of cells. AMC303 might directly or indirectly induce cell death, leading to a decrease in the number of clonogenic cells. Treatment of CRC cells with AMC303 showed a slight decrease of the colony forming ability of the cells compared to HGF alone, while cells exposed to AMC303 reduced the size of colonies (**Figure 3B-C**).

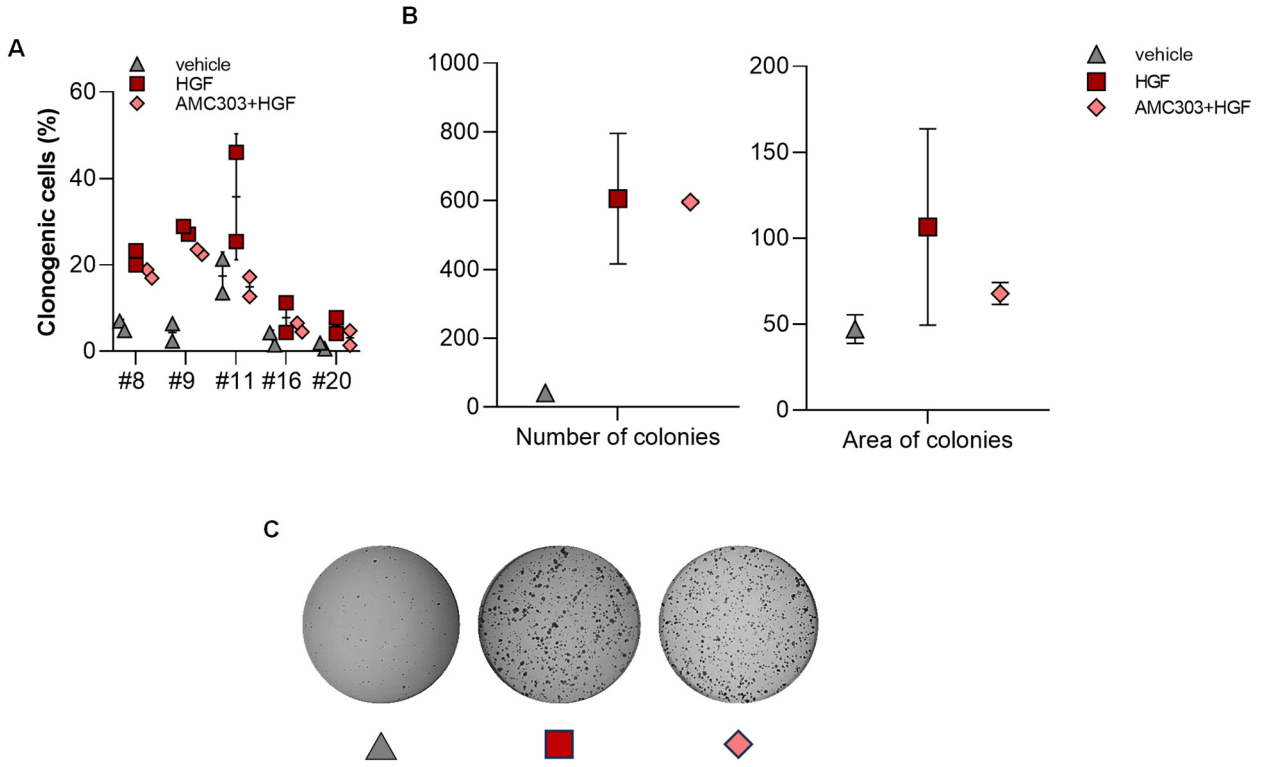


Figure 3. A. Clonogenic assay in CMS2 CRC cells treated with HGF alone or in combination with AMC303 over 21 days. **B.** Quantitative analysis and **C.** the images of colony forming assay

Based on the initial findings that AMC303 exhibited, with a partial efficacy in reducing cancer cell viability, a thorough review of the literature was conducted to identify potential pathways contributing to the observed resistance. Several studies have implicated the PI3K/Akt/mTOR and MAPK/ERK pathways in cancer cell survival and proliferation, as well as in resistance to various chemotherapeutic agents. Therefore, inhibitors targeting these pathways were selected for combination therapy. By combining AMC303 with inhibitors targeting the PI3K/Akt/mTOR and MAPK/ERK pathways, we hypothesized that a more effective suppression of cancer cell survival and proliferation could have been achieved, potentially overcoming the resistance observed with AMC303 alone. (PMID: 11687500, PMID: 16847462). To this aim, we chose c-Met downstream pathways inhibitors including Copanlisib (PI3K inhibitor), Apitolisib (dual PI3K/mTOR inhibitor), Ipatasertib (AKT inhibitor), Trametinib (MEK inhibitor), Crizotinib (HGFR inhibitor), and C188-9 (STAT3 inhibitor).

For determination of toxicity of the selected inhibitors, CRC cells were seeded in 96 well plate and treated with different concentrations, then cell titer glo was read over 72 h and a viability above 80% was acceptable as the highest non toxic concentration for each inhibitor (**Figure 4A**). In the next step, the highest non-toxic concentration of each inhibitor was selected, and the CRC cells were treated with them in presence of HGF, then western blot was performed and the expression level of proteins involved in MAPK and PI3K pathways were analyzed (**Figure 4B**). Apitolisib decreased the expression level of all the proteins compared to HGF alone and vehicle.

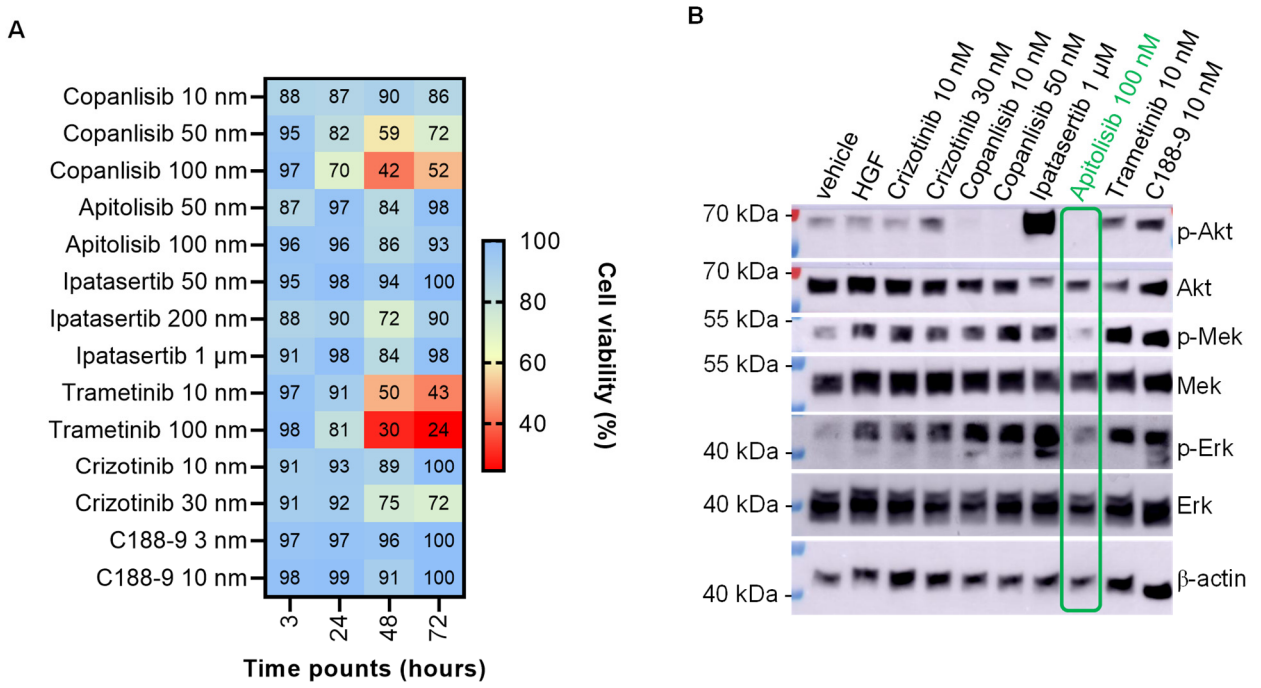


Figure 4. A. The percentage of viable cells after treatment with different concentrations of TRKIs. **B.** The expression level of P-MEK , MEK, P-AKT, and AKT, P-ERK , and ERK were analyzed by western blotting. Beta Actin was endogenous control.

Then the effect of Apitolisib on cell viability was analyzed by cell proliferation assay. To this aim, CRC cells were treated with Apitolisib alone or in presence of HGF, then cells were counted in a period of 10 days (**Figure 5A**). Moreover a colony forming assay was conducted and depicted that Apitolisib alone is not able to suppress the effect of HGF completely (**Figure 5B**).

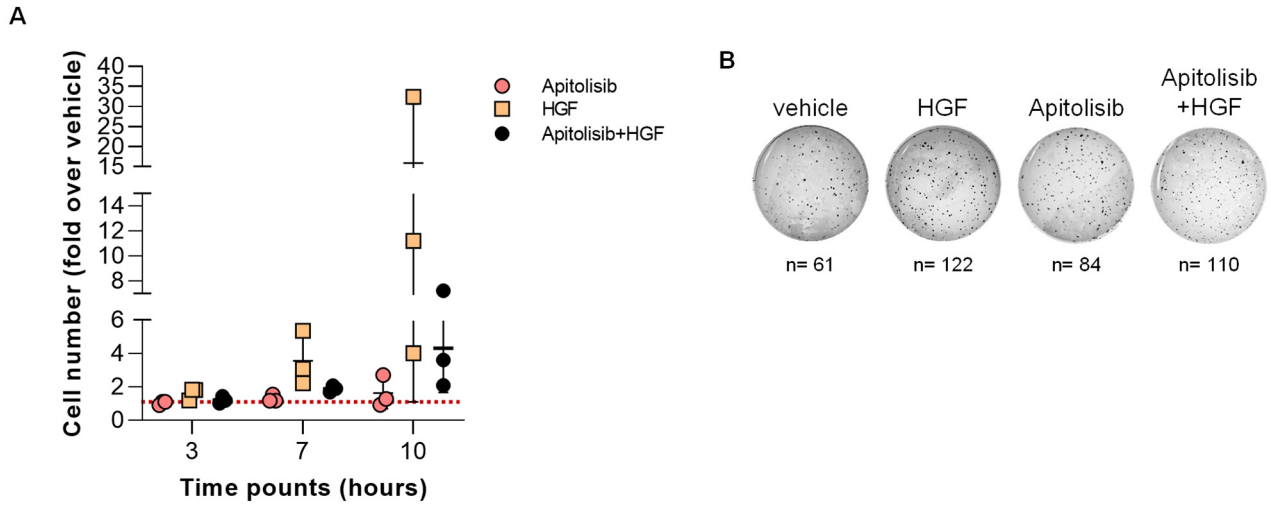


Figure 5. A. Cell proliferation assay analyzed by row counting in 10 days. **B.** Colony forming assay over 21 days.

Furthermore, as a combinatorial treatment CRC cells were treated with AMC303 alone or in combination with Apitolisib, and exposed to HGF, then cell counting, clonogenic assay, and colony forming assay were performed (**Figure 6**). The result showed that, although AMC303 and Apitolisib alone are not sufficient for suppressing the effect of HGF on clonogenic potential and proliferation of cells, their combination hamper the effects of HGF. As the combinatorial treatment was able to reduce the number and area of colonies in colony forming assay, also the clonogenic potential of cells was depleted. Additionally, to analyze the downstream pathways, western blot was performed to investigate the expression of proteins associated with Beta Catenin, MAPK, PI3K, JAK/STAT3 pathways (**Figure 7**). Treatement with AMC303 alone exhibited a reduction in non-phospho β-catenin expression compared to the HGF-treated group. However, the combination of AMC303 and Apitolisib resulted in a further reduction in non-phospho β-catenin expression, indicating a synergistic effect. These results suggest that combinatorial treatment may be a more effective strategy for inhibiting Wnt signaling in these cells.

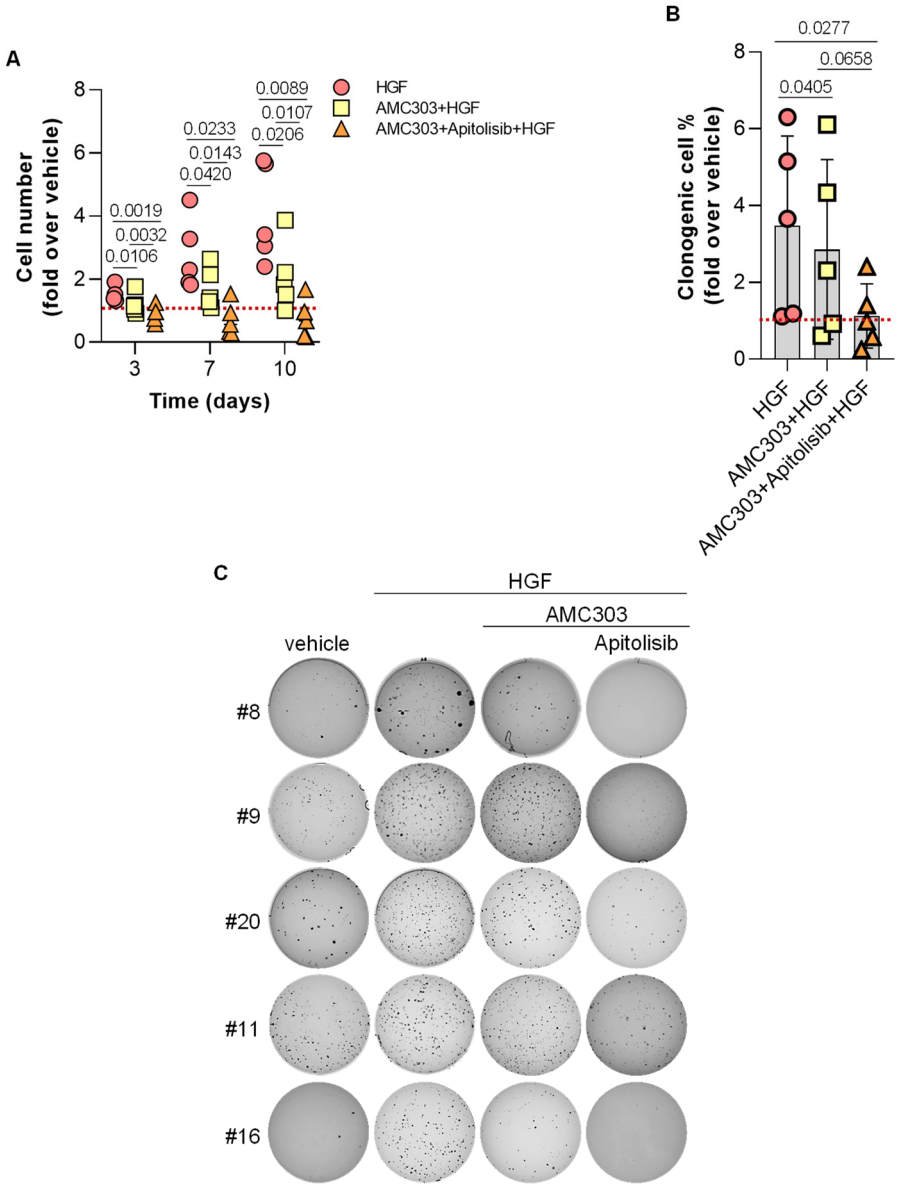


Figure 6. A. Row counting of CRC cells following the treatment with AMC303 alone or in combination with Apitolisib in presence of HGF over 10 days. **B.** Clonogenic assay of pretreated cells by AMC303 and Apitolisib which are exposed to HGF. **C** Colony forming assay over 21 days.

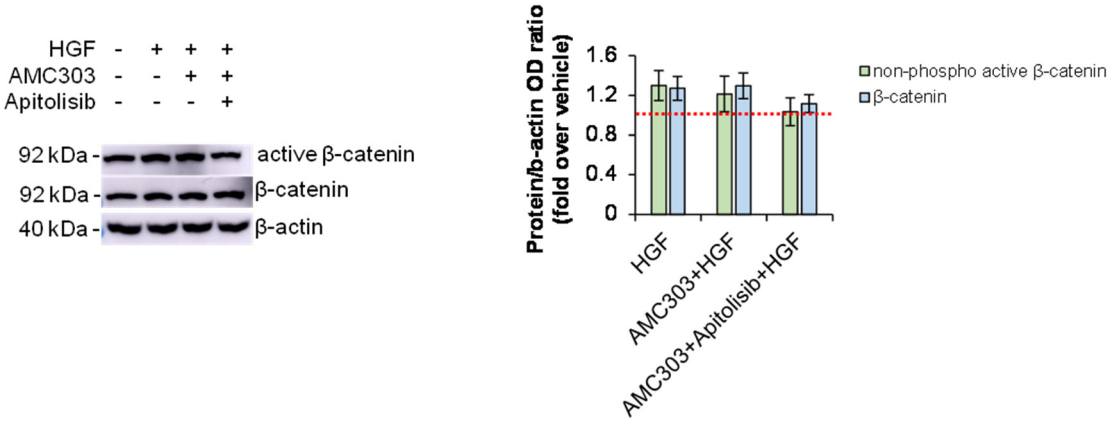


Figure 7. Comparison between the effects of AMC303 alone and combination of AMC303 and Apitolisib on the expression of non-phospho β-catenin and phospho β-catenin using western blot**.**

Next, to visualize the differences in gene expression, a heat map was generated (**Figure 8**), illustrating the relative expression levels across all samples analyzed. The heat map highlights the most significantly upregulated and downregulated genes, providing an intuitive overview of the differential expression patterns. explored the expression level of genes associated with EMT, stemness, and chemoresistance in groups treated as: vehicle, HGF, HGF + AMC303, and AMC303+ Apitolisib in presence of HGF. Our results, based on the expression of genes associated with CSCs and EMT show that CR-CSphCs treated with AMC303 + Apitolisib cluster similarly to those exposed to vehicle (**Figure 8**).

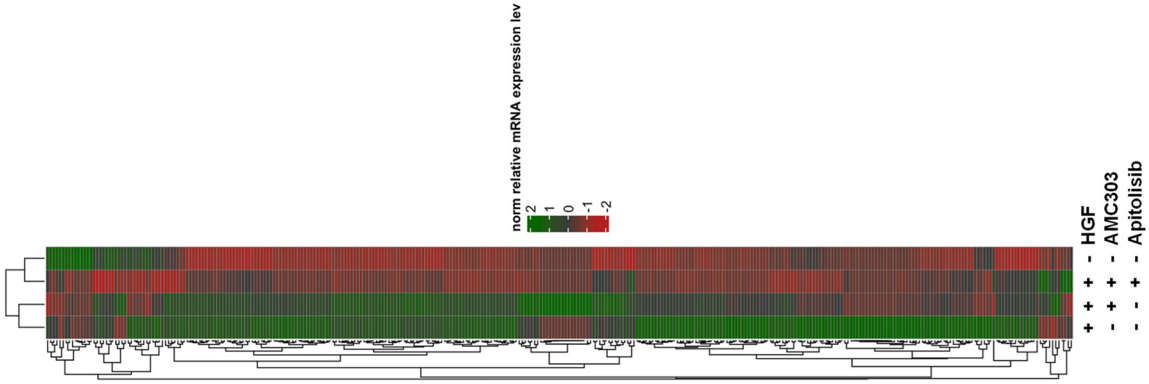


Figure 8. Array for mRNA expression of genes associated with EMT and CSCs, in CRC cells treated by Apitolisib, and AMC303 in presence of HGF.

Discussion

This study aimed to elucidate the effects of AMC303 and Apitolisib on CSCs in the presence of HGF. Our findings reveal that treatment with AMC303 alone did not significantly inhibit the proliferative effects of HGF on cancer stem cells. Specifically, assays assessing cell proliferation, clonogenic potential, and colony formation demonstrated that HGF continued to enhance these characteristics, indicating that AMC303 may not sufficiently antagonize the growth-promoting

signals mediated by HGF. Similarly, treatment with Apitolisib mitigates the effects of HGF partially. This observation suggests that while both compounds may possess therapeutic potential in other contexts, they do not independently disrupt the HGF-mediated pathways that facilitate cell proliferation and clonogenicity. In contrast, a significant alteration was observed when cancer stem cells were treated with a combination of AMC303 and Apitolisib. This combinatorial approach markedly inhibited the effects of HGF, suggesting a synergistic interaction between the two compounds. The enhanced efficacy of this combination treatment implies that AMC303 and Apitolisib may target distinct molecular pathways within the HGF signaling cascade, thereby providing a more effective strategy for counteracting HGF-mediated tumorigenesis. These results underscore the complexity regarding targeting cancer stem cells and highlight the potential advantages of combination therapies in overcoming growth factor-mediated resistance. Future investigations should focus on delineating the specific molecular mechanisms underlying this synergistic effect and evaluating the therapeutic efficacy of this combination in vivo to assess its clinical relevance. Recent research has increasingly demonstrated that targeting the HGF/c-MET signaling pathway is a promising therapeutic approach for treating various human cancers. This strategy has shown effectiveness in inhibiting the growth of multiple cancer types, including non-small cell lung cancer, hepatocellular carcinoma, gastric cancer, colorectal cancer, ovarian cancer, bladder cancer, head and neck cancer, and cervical cancer [109-116]

Dysfunction in HGF/c-Met signaling has been linked to cell growth, tumor progression, and metastatic traits across various cancer types, indicating its potential as a novel therapeutic target. While c-MET activation typically occurs transiently during normal physiological processes, it may become persistently active during the initiation and advancement of tumors. The activation of c-MET pathways in tumor cells during progression facilitates their detachment from neighboring cells, leading to the degradation of the basement membrane, which enhances cellular mobility and increases the risk of metastasis [117]. CD44v6 has been identified recently as both a functional biomarker and a potential therapeutic target for metastatic cancer-initiating cells by activating the PI3K signaling pathway [118]. It is noteworthy that the PI3K pathway has been shown to play a significant role in breast cancer development, with deregulation occurring in nearly 50% of patients [119]. The PI3K pathway is viewed as a promising biological target, particularly for the treatment of the most aggressive tumors [120]. Deregulation of the PI3K/Akt/mTOR pathway is often implicated in the initiation, progression, and metastasis of CRC, and it also contributes to drug resistance [121]. Targeting CD44v6 completely inhibited the invasive ability of tumorigenic CRC, suggesting that their metastatic potential relies on signaling pathways activated by HGF through CD44v6 and c-MET. These receptors work together to stimulate the PI3K/AKT pathway, which facilitates migration and survival signaling in CRCs. CD44v6+ cells exhibit increased activation of the PI3K/AKT pathway, which is initiated by ligand binding and the epigenetic silencing of PTEN, a characteristic feature of colorectal cancer stem cells [122].

In a study, results indicated that both a CD44v6-specific peptide and a CD44v6 antibody can effectively inhibit c-Met activation in endothelial cells, thereby impairing HGF-induced migration and tubular vessel formation by these cells. Notably, this peptide and antibody also obstructed VEGF-induced activation of VEGFR-2. Furthermore, ERM proteins associated with CD44 regulate signaling from VEGFR-2. Thus, the mechanism by which CD44 functions appears to be quite similar for the activation of both c-Met and VEGFR-2 [123].
so, targeting CD44v6 completely inhibited the ability of tumorigenic CRCs, suggesting that their metastatic potential relies on signaling pathways activated by HGF through CD44v6 and c-MET. These receptors work together to stimulate the PI3K/AKT pathway, which facilitates migration and survival signaling in CR-CSCs. AMC303 binds with high affinity to the ectodomain of CD44v6.

Recent studies have shown that AMC303 inhibits the activation of the receptor tyrosine kinases c-MET and RON allosterically in various epithelial tumor cells, as well as VEGFR-2 in endothelial cells, through its extracellular binding to CD44v6.
Recent studies have shown that PI3K inhibitors are likely more effective in cancers with mutations in the PI3K pathway, including CRC [124, 125]. Therapeutic targeting of the PI3K pathway using small molecule inhibitors may offer clinical advantages, either as standalone treatments for cancers that are dependent on PI3K or more broadly in combination with other conventional or targeted therapies. Several inhibitors aimed at the PI3K pathway have now progressed into clinical trials [102, 126, 127].

Apitolisib (GDC-0980) is a dual inhibitor of PI3K and mTOR that can trigger apoptosis in cancer cells and suppress their growth. Apitolisib has been reported to demonstrate a wide range of antitumor activity across various cancers and has been utilized in Phase I/II studies for the treatment of solid tumors. The most significant effects were noted in breast, prostate, and lung cancer cell lines, while the impact was less pronounced in pancreatic cancer and melanoma cell lines. In a preclinical study, GDC-0980 effectively inhibited the PI3K pathway and reduced tumor cell viability across a wide range of cancer cell lines. Mutations in BRAF or RAS were found to be negative predictors of the drug's effectiveness in all tumor types examined [128, 129]. A study investigates the effects of combining 5-FU with thymoquinone (TQ) on CR-CSCs The research demonstrates that the combination significantly enhances cytotoxicity against CSC populations while effectively inhibiting the WNT/β-Catenin and PI3K/AKT signaling pathways. The findings suggest that this combinatorial approach can target multiple oncogenic pathways, leading to improved eradication of CRCs compared to single-agent therapies. This aligns with our findings that simultaneous targeting of c-MET/CD44v6 interactions and PI3K signaling can lead to a more substantial reduction in CR-CSC proliferation and clonogenic potential [130].

The combination therapy approach we investigated presents a promising strategy for enhancing therapeutic efficacy against colorectal cancer. Traditional treatments often fail to eliminate CR-CSCs, leading to treatment resistance and disease recurrence. By concurrently targeting c-MET/CD44v6 interactions and PI3K signaling, we may be able to more effectively eradicate these resilient cell populations. The results of this study suggest that while neither compound alone sufficiently counteracts HGF's effects on CR-CSCs, their combined use effectively diminishes the proliferative capacity of these cells. This indicates that simultaneous targeting of multiple pathways may be necessary to overcome the robust mechanisms that CR-CSCs employ to evade treatment. The promising results from our study warrant further investigation into the molecular mechanisms underlying the observed effects. Future research should focus on elucidating how the combination therapy influences signaling pathways beyond PI3K and c-MET/CD44v6 interactions, potentially identifying additional targets for intervention. Additionally, clinical trials will be essential to validate these findings in patient populations and assess the safety and efficacy of this combinatorial approach. In conclusion, combining a c-MET/CD44v6 inhibitor with a PI3K inhibitor represents a novel strategy that could significantly improve treatment outcomes for patients with colorectal cancer by effectively targeting CSCs and overcoming chemoresistance. As we continue to explore this therapeutic avenue, we may pave the way for more effective interventions in managing this challenging disease. This expanded section incorporates insights from recent studies on combination therapies, emphasizing their relevance to colorectal cancer treatment while aligning with your research findings.

CONCLUSIONS

A subset of tumor cells known as cancer stem cells (CSCs) has the ability to initiate tumors and trigger recurrences. CSCs come from either differentiated cells or adult tissue-resident stem cells at the time of tumor onset. The improved capacity of CSCs to induce tumor growth, proliferate, migrate, and withstand treatment actions is another characteristic. This suggests that CSCs play a significant part in the genesis of cancer and makes them a target for anti-cancer therapies. In a variety of cancers, CD44v6 is a particular marker on cancer stem cells (CSCs) that is connected to tumor growth, invasion, and resistance to treatment. The ability of CSCs to multiply, migrate, and avoid therapy is improved by CD44v6 through the activation of important survival pathways such as MET, MAPK/ERK, and PI3K/AKT. Through the targeted elimination of CSCs, CD44v6 may enhance cancer treatments by decreasing the risk of metastasis and recurrence. CD44v6 is therefore a prospective target for new therapies to control cancer over the long term. Cancer treatment is made extremely difficult by CSCs' resistance to conventional treatments like chemotherapy and radiation. This resistance results from a number of processes that enable CSCs to avoid the cytotoxic effects that normally kill non-stem cancer cells, such as increased DNA repair capacity, and survival pathway activation. Therefore, CSCs can survive and cause tumor recurrence and metastasis even after an initial good response to treatment. It is essential to target these resistant CSCs in order to increase the effectiveness of cancer treatments and provide patients with long-term remission. Natural compounds have drawn interest because of their potential to efficiently target cancer stem cells (CSCs), providing an alternative to conventional cancer treatments. Many natural compounds can specifically interfere with CSC signaling pathways, preventing their self-renewal, proliferation, and resistance mechanisms. Furthermore, natural compounds frequently possess immunomodulatory, anti-inflammatory, and antioxidant qualities that might lessen inflammation that promotes tumor growth and enhance the tumor microenvironment's reaction to treatment. These substances increase the effectiveness of treatment and lower the risk of recurrence and metastasis by sensitizing CSCs to chemotherapeutic or radiotherapeutic treatments. (**Chapter1**). For instance, extracts from artichokes and olives have encouraging results against cancer stem cells, which could improve the results of cancer treatment. Olive and artichoke extracts may help prevent recurrence and metastasis by reducing CSC proliferation, inducing apoptosis, and increasing susceptibility to treatment, according to studies. Patients with colorectal cancer may benefit from this natural, focused approach in addition to traditional treatments. Natural compounds have shown promise in the treatment of cancer, despite potential risks and negative effects. Variations in potency and purity can lead to unpredictable results, even if some chemicals may reduce the effectiveness of traditional treatments. For instance, antioxidants can counteract the effects of chemotherapy and radiation, and high doses of substances like curcumin can damage the liver. Additionally, many herbs affect the way medications are metabolized, which could make them more toxic or less effective. Standardized dosages and close observation are essential to lowering these risks when using natural substances to treat cancer (**Chapter2**).

Targeted therapy for CSCs provides accuracy in targeting particular cancer pathways, potentially minimizing side effects and relapse risk. Additionally, this method can increase susceptibility to common therapies like chemotherapy. Long-term efficacy may be limited by CSC resistance, and the requirement for exact target identification makes the development of these treatments difficult and expensive. Furthermore, there is still a chance of unforeseen consequences for non-CSC cells, and not all CSCs have distinct markers, making universal application more difficult. Targeted CSC therapy has many obstacles in spite of its potential. The goal of targeted therapy for CD44v6 is to prevent the over expression of this CD44 glycoprotein variation in CSCs. To increase sensitivity to traditional treatments, small-molecule inhibitors that target the pathways that CD44v6 activates, such as PI3K/AKT and MAPK/ERK, are also being investigated. These treatments aim to reduce the number of CSCs and enhance patient outcomes by focusing on CD44v6, but there are still issues including off-target effects and the requirement for additional research. A promising strategy to fight CSCs and improve treatment effectiveness is targeting both CD44v6 and c-MET at the same

time. While c-MET is a receptor tyrosine kinase that is essential for cell invasion and proliferation, CD44v6 is frequently overexpressed in CSCs, which aids in tumor growth and metastasis. This dual strategy attempts to interfere with several signaling pathways implicated in tumor growth and CSC characteristics by utilizing a mix of monoclonal antibodies and small-molecule inhibitors that precisely target both CD44v6 and c-MET. By focusing on both indicators, the constraints presented by each target separately may be addressed and CSC viability, metastatic potential, and sensitivity to traditional therapy may all be enhanced (**Chapter3**). One therapeutic strategy for targeting CSCs, is to block the PI3K (phosphoinositide 3-kinase) pathway. By interfering with CSC signaling, inhibitors that target this route can decrease the cells' survival and increase their vulnerability to traditional treatments. Although this strategy has the potential to improve treatment effectiveness, issues like possible adverse effects and the possibility of CSC adaptability call for more study to maximize these focused tactics. Colorectal CSCs can be reprogrammed by tumor microenvironment growth factors, including HGF that is released by CAFs. In this scenario, AMC303 treatment alone is not able to prevent the effects mediated by HGF in terms of proliferation, clonogenic and colony forming potential of CR-CSCs. This effect could be driven by the activation of parallel pathways, such as MAPK, PI3K, and JAK/STAT. Interestingly, Apitolisib showed a great inhibitory effect on all these pathways, using non-toxic concentration. Indeed, the combinatorial regimen based on Apitolisib and AMC303 was sufficient to impair the HGF-driven CR-CSCs reprogramming, thus suggesting its putative clinical application to prevent or treat the metastatic disease in CRC patients.

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