PhD Course in

"TRANSLATIONAL MEDICINE AND FOOD: INNOVATION, SAFETY AND MANAGEMENT" - *XXXIII Cycle*

Coordinator: Prof. Matteo Alessando del Nobile

PhD thesis

Expression analysis of LASP1 in human cancer cell lines, role of Src signaling pathways in endothelial cell permeability and natural compounds in chemoprevention

PhD Student: Mariia Zadorozhna Mariia Zadorozhna

Tutor: Prof. Lorenzo Lo Muzio

Co-tutor: Prof. Domenica Mangieri Domenica Mangieri

Personal Data

Name: Mariia Zadorozhna

Born in: Chernivtsi (UKR)

Nationality: Ukrainian

Language: Ukrainian, Italian, English, Russian

Address: via N. Patierno 6, Foggia, Italy

Telephone: +393703555348

Email: mariia.zadorozhna@unifg.it

Premise

The research activity consists of 3 main parts:

The first part was focused on the correlation between the LASP1 expression and the level of malignancy of cancer. The activities were represented by *in vitro* experiments on human breast cancer cells (MDA-MB-231, SK-BR-3 and MCF-7), human oral squamous cell carcinoma cell lines (CAL-27, HSC-2) and human gynaecological leiomyosarcoma cell line (SKUT-1). All activities were supervised by my tutor prof. Lorenzo Lo Muzio and co-tutor Prof. Domenica Mangieri (University of Foggia).

The second part of my research study was conducted in Rudbeck laboratory, Uppsala University, Sweden and was focused on the study of Src family kinases (SFKs) in the vascular biology. The experimental activities were done *in vitro* in endothelial cell line, Human Umbilical Vein Endothelial Cells (HUVECs). All the activities were supported by prof. Lena Claesson-Welsh and Ph.D. Yi Jin (Rudbeck laboratory, Vacular Biology, Uppsala University, Sweden).

The third part of research study was concentrated on the role of natural compounds in chemoprevention/chemotherapy. To structure and highlight all studied information, I did the literature overview of two natural compounds: piperine and ginger.

Pubblications

Zadorozhna M. The achievements in the sphere of stem cells. *BIMCO Khyst 2013*, 16, 123.

Zadorozhna M, Gutsuliak N, Huzyk N. Radionuclide method of tuberculosis diagnosis. *BIMCO Khyst 2015*, 18, 311.

Zadorozhna M, Gutsuliak N., Huzyk N., Bilous I. Indicators of antioxidant blood system in patients with diabetic polyneuropathy. *BIMCO Khyst* 2015, 18, 111.

Andriiv J, **Zadorozhna M**, Huzyk N. Dysfunctional uterine bleeding in women with premenopausal. *BIMCO Khyst* 2015, 18.

Khrunyk L, **Zadorozhna M**, Huzyk N, Voloshynska K. Particularities of cardiometabolic risk in premenopausal women. *BIMCO Khyst* 2014, 17, 137.

Zadorozhna M, Tataranni T, Mangieri D. Piperine: role in prevention and progression of cancer. *Mol bio rep 2019*. https://doi.org/10.1007/s11033-019-04927-z.

Zadorozhna M, di Gioia S, Conese M, Domenica M. Neovascularization is a key feature of liver fibrosis progression: anti-angiogenesis as an innovative way of liver fibrosis treatment. Mol bio rep 2020. DOI 10.1007/s11033-020-05290-0

Zadorozhna M, Mangieri D. Mechanisms of Chemopreventive and Therapeutic Proprieties of Ginger Extracts in Cancer. Int. J. Mol. Sci. 2021, 22, 6599. https://doi.org/10.3390/ijms22126599

Part 1

1. Expression analysis of LASP1 in human cancer cell lines

Introduction

Cancer takes place by malignant transformation of normal cells; it remains one of the most common causes of death worldwide [1]. In fact, the survival rate for patients suffering from tumors in developed countries remains very poor, despite advances in diagnosis, surgery, radiation, chemotherapy as well as target therapy [2]. Generally, cancer onset and progression are complex phenomena that involve numerous steps and a number of signaling pathways [3]. LIM and SH3 protein (LASP1), is an actin-binding protein. It is believed that this protein plays a crucial role in cancer dissemination, progression, metastasis, and angiogenesis [4]. Several studies documented that LASP1, ubiquitously expressed in cells [5, 6], results up-regulated in numerous different tumor entities; it seems to be involved in several cues of tumorigenesis of several cancers including gallbladder cancer, human non-small-cell lung cancer (NSLC), colorectal cancer (CRC), ovarian and breast cancers, and choriocarcinoma [4, 7-12]. Thus, LASP1 appears to exert a driving role in regulating cancer cell metastatic propensity, probably perturbing the architecture and dynamics of focal adhesion, triggering cell migration, and invasion facilitating the dissemination of tumors.

Cancer strongly interacts with the surrounding microenvironment including stromal cells (endothelial cells), pericytes (neovascular component), fibroblasts and ECM molecules. All these factors contribute to establish the fate of neoplastic cells [3]. Interestingly, both tumor masses and surrounding microenvironment are often characterized by hypoxia that, in turn, stabilizes HIF-1α, which in form of heterodimeric transcription factor, acts on target genes expression [13]. Several studies documented that hypoxia is, in part, responsible for induction of epithelial-mesenchymal transition (EMT) in various carcinoma [14]. EMT is a positive phenomenon of tumor dissemination, however, the mechanisms implicated in cytoskeleton dynamic during this event are not very clear. Interestingly, LASP1 is known to be a target of HIF-1α [15], this aspect allows to suppose that LASP1

may have a crucial role during EMT and consequent cancer aggressiveness. As matter of fact, Salvi and co-workers demonstrated that hepatocellular carcinoma overexpressed Vimentin, a key EMT marker, by means of LASP1 [16]. Moreover, experimental data demonstrated that in severe tumor circumstances, LASP1 can break its linkage with cytoskeleton, reinforcing its binding with the tight junctions (TJs) protein ZO2, and, as dimer LASP1-ZO2, moves to the nucleus [17]. Remarkably, the molecular structure of LASP1 contains a LIM cysteine-rich domain (N-domain) composed of two zinc fingers (**Fig. 1**), thus it is conceivable that the protein can bind directly the DNA acting as a transcriptional factor and/or as a regulator of target genes including angiogenic and proteases genes, both implicated in cancer dissemination.

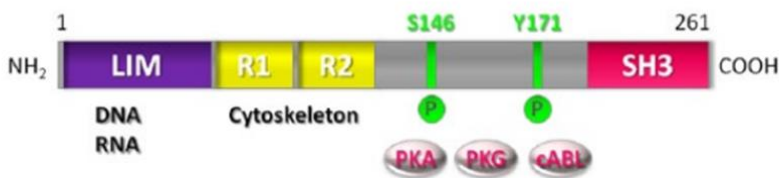


Figure 1. **Schematic representation of LASP1 domain structure.** The N-terminal portion is a LIM cysteine-rich domain composed of two zinc-finger residues. LIM domain is followed by two 35-residue nebulin-like segments, repeated in tandem, denominated, respectively, R1 and R2 domains. R1/R2 domains are flanked by a phosphorylation motif (Ser-146 and Tyr-171). The C-terminal is a SRC homology region 3 (SH3) domain. Modified from Ruggieri et al [6].

The main activity consisted in working with different tumor cell lines and finding the correlation between the LASP1 expression and the level of malignancy of cancer and its contribution to EMT and the consequent ability of tumor to disseminate, that augments its aggressiveness (**Fig. 2**).

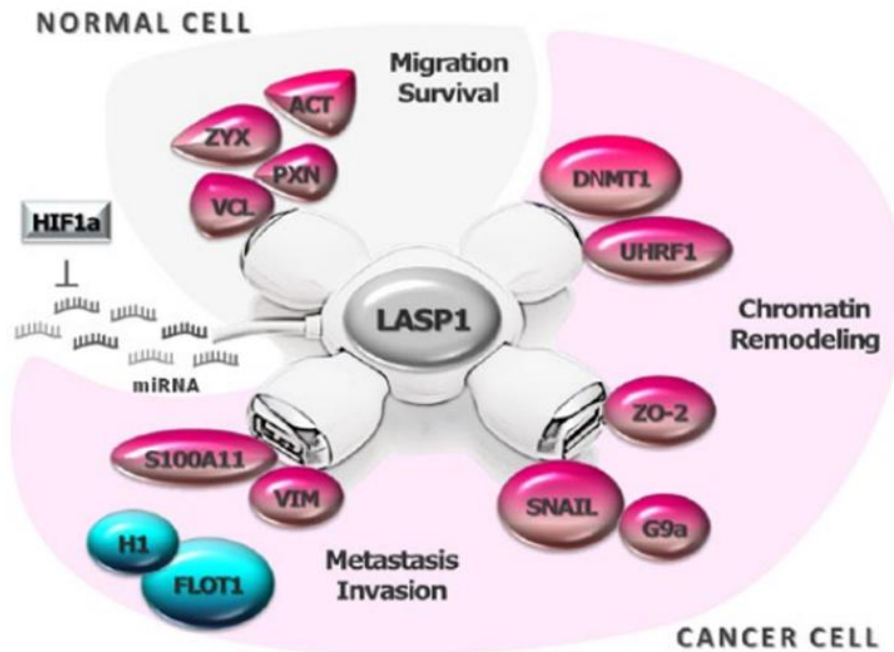


Figure 2. LASP1 functions in the pathological processes of cancer cell. LASP1 actively contributes to tumor aggressiveness by promoting cell proliferation, metastasis dissemination, and chromatin remodelling through the direct and indirect interactions with several proteins. Moreover, LASP1 oncogenic activity is controlled by several miRNAs which are modulated by HIF-1α. LASP1 interacting proteins are indicated. The direct and indirect binding partners are indicated in deep pink and light blue, respectively. *ZYX*: zyxin; *PXN*: paxillin; *VCL*: vinculin; *ACT*: actin; *VIM*: vimentin; *S100A11: S100* calcium-binding protein A11; *HI*: histone H1; *FLOT1*: flotillin-1; *ZO-2*: zonula occludens 2; *DNMT1: DNA* methyltransferase 1; *UHRF1*: ubiquitin-like with PHD and ring finger domain 1; *G9a*: histone methyltransferase G9a; *SNAIL*: snail family transcriptional repressor 1; *HIF-1α*: hypoxia-inducible factor 1-alpha subunit. Modified from Ruggieri et al [6].

As far as hypoxia, being the “hallmark of cancer”, controls both directly and indirectly the expression of specific genes including LASP1, I have studied the viability of cancer cells in different hypoxic conditions, and I was establishing a hypoxic model for MDA-MB-231 cells *in vitro*.

Material and Methods:

Cell cultures

Human breast cancer cell lines (MDA-MB-231, SK-BR-3 and MCF-7) were cultivated in Dulbecco's Modified Eagle Medium High Glucose (DMEM HG) (GIBCO^{TM}) containing L-Glutamine 4mM (GIBCO^{TM}), 100 U/mL penicillin and 100 mg/mL streptomycin supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO^{TM}) in a humidified chamber of 5% CO_2 at 37°C. Human oral squamous cell carcinoma cell line (CAL-27) and a human gynaecological leiomyosarcoma cell line (SKUT-1) were cultured with DMEM Low Glucose (DMEM LG) (GIBCO^{TM}) supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin (GIBCO^{TM}), 10% FBS (GIBCO^{TM}) in a humidified chamber of 5% CO_2 at 37°C. HSC-2, another type of human oral squamous cell carcinoma cell line was grown in Roswell Park Memorial Institute 1640 (RPMI-1640) (GIBCO^{TM}) supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin (GIBCO^{TM}), 10% FBS (GIBCO^{TM}) in a humidified chamber of 5% CO_2 at 37°C.

The above-mentioned cell lines were cultured at 30, 50 and 80% of confluence in the Petri dish (low, medium, and high density, respectively).

Cell lysates and protein dosage

The cells were scripped and the cell lysates were prepared by solubilization in Tris-HCl buffer, pH 7.5, containing 1% Igepal CA-630 (Spectrum chemical), 150 mM NaCl and proteinase inhibitor (100:1). The protein content of cell lysates was measured with Bradford method [18] and the samples were stored at -80°C until use.

Western blotting

The lysates were resolved by SDS-PAGE on an 12% gradient gel, 25µg of proteins was loaded in each lane. Proteins were then transferred to polyvinylidene fluoride (PVDF) membrane using the BIORAD Trans-Blot® Turbo™ Blotting System. The saturation of membrane was performed with

10% of dry milk diluted in Tween-Tris-buffered saline (TTBS) for 1 hour at 37°C. Consecutively, the incubation using the specific primary rabbit anti-human LASP-1 antibody (ABCAM; code ab156872,) diluted in TTBS (1:20000) was done and was left overnight. The next day, after 3 washes in TTBS, the membrane was incubated with a goat anti-rabbit peroxidase-conjugated secondary antibody (Thermo Fisher Scientific; code 31460) diluted in TTBS (1:20000). Signals were visualized using the BIORAD ChemiDoc™ MP Imaging System. After that the stripping of membrane with a NaOH 0,25M was performed, the saturation in 10% dry milk was repeated for 1 hour on 37°C. Following, the incubation with the mouse anti-human beta-actin primary antibody diluted in Tris-buffered saline (TBS) (1:10000) was left overnight. The next, day after 3 washes in TBS, the incubation with anti-mouse peroxidase-conjugated secondary antibody diluted TBS (1:2500) was performed. Signals were visualized using the BIORAD ChemiDoc™ MP Imaging System.

Chemically induced hypoxia and viability analysis assay

CoCl2 is often used to simulate a hypoxic environment for cell cultures [19]. In this study, confluent MDA-MB-231 cell line was cultured in DMEM with various concentrations of CoCl2 (50µM, 100 µM, 200 µM) (Alfa Aesar™) and for different time duration (24h and 48 h). The cell viability analysis was performed by using trypan blue assay.

Results and discussion

In this study I first investigated the expression of LASP1 in a wide spectrum of cancer cells (**Tab. 1**), then I studied the effect of a hypoxic environment on the cell viability and on the biological behavior focusing on breast cancer MDA-MB-231 cells due to this cell line aggressiveness.

Table 1. Cell lines studied and their main characteristics.

Cell line	Main characteristics
MDA-MB-231	Cell line derived from human mammary gland/breast; adenocarcinoma; originated from metastatic site: pleural effusion; highly aggressive, invasive and poorly differentiated triple-negative breast cancer (TNBC).
SK-BR-3	Cell line derived from human mammary gland/breast; adenocarcinoma; originated from metastatic site: pleural effusion; over-expresses the HER2 gene product.
MCF-7	Cell line derived from human mammary gland/breast; adenocarcinoma; originated from metastatic site: pleural effusion; cells have characteristics of differentiated mammary epithelium.
CAL-27	Poorly differentiated human squamous cell carcinoma at the middle of the tongue; cells were resistant to treatment with VDS (vindesine sulfate), CDP (cis-platinum) or ACTD (actinomycin D).
HSC-2	Human oral cavity squamous cell carcinoma; cell line has neither invasive nor metastatic potential.
SKUT-1	Uterine corpus leiomyosarcoma; cell line has a moderate invasive properties.

The information about cell lines was taken from https://www.lgcstandards-atcc.org/?geo_country=it and http://cellbank.nibiohn.go.jp/english/.

As LASP1 is predominantly involved in the reorganization of cytoskeleton during cellular motility, the expression of LASP1 within the cells of the same cell line in different degrees of confluence was monitored (**Fig. 3-5**).

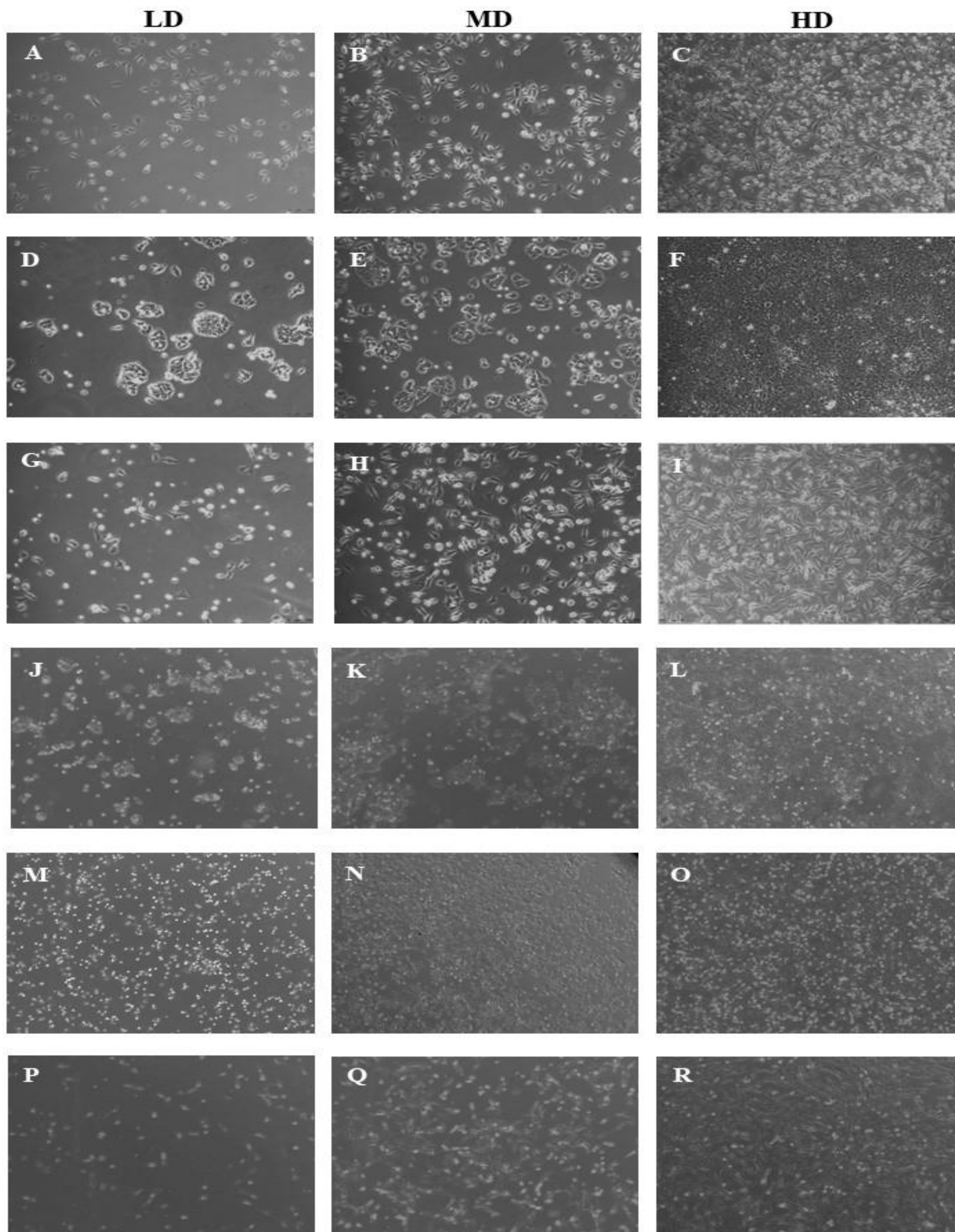
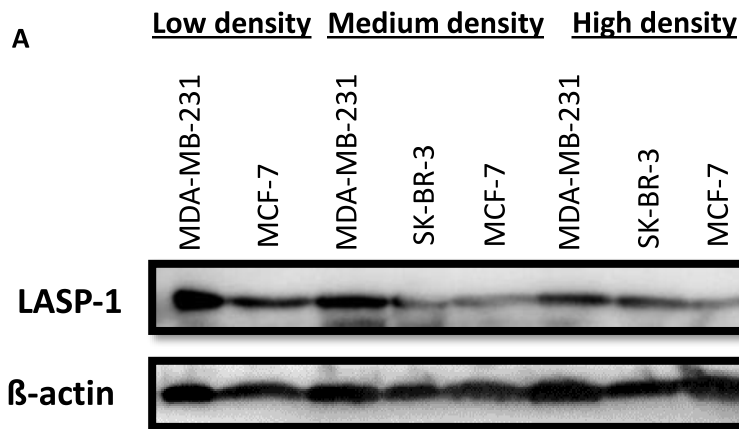


Figure 3. **Phase-contrast imaging for demonstration low, medium, and high densities of cell lines.** MDA-MB-231 (panels A, B, C); MCF-7 (panels D, E, F); SK-BR-3 (panels G, H, I); CAL-27 (panels J, K, L); HSC-2 (panels M, N, O); SKUT-1 (panels P, Q, R). The microscope effective magnification is 10x.



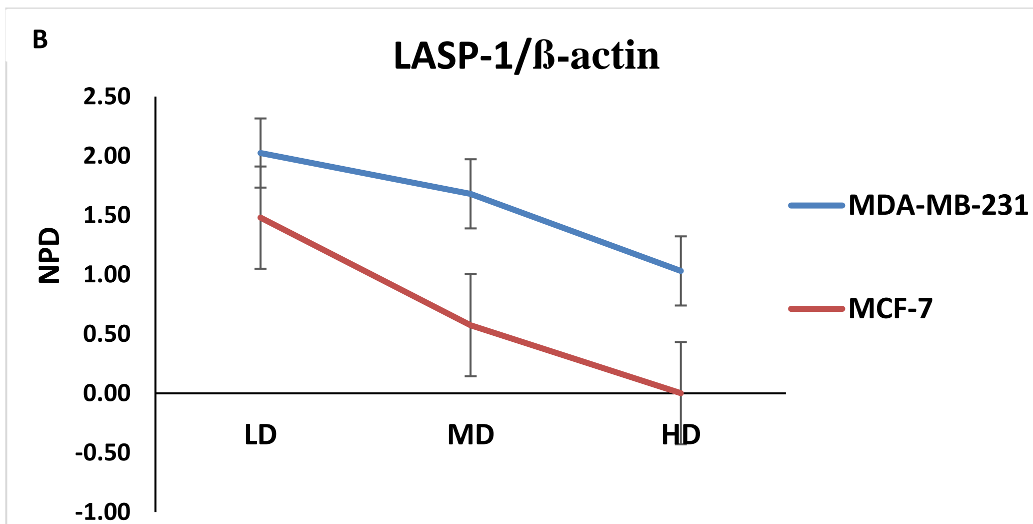
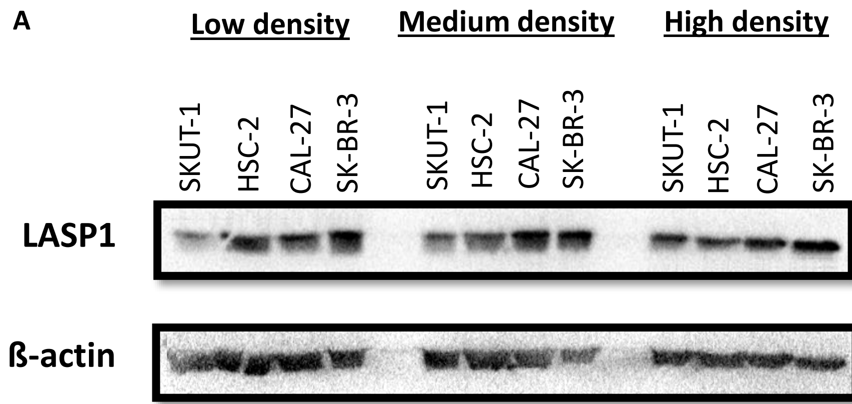


Figure 4. Analysis of LASP1 expression in different human breast cancer cells. (**A**) Representative western blot reflecting LASP1 protein level in MDA-MB-231, SK-BR-3, MCF-7 cell lysate; β-actin was used as a western blotting loading control. Signals were visualized using the BIORAD ChemiDoc™ MP Imaging System. (**B**) Normalized LASP1 expression (Normalized pixel density (NPD) within cell lines MDA-MB-231 and MCF-7 cell lines.



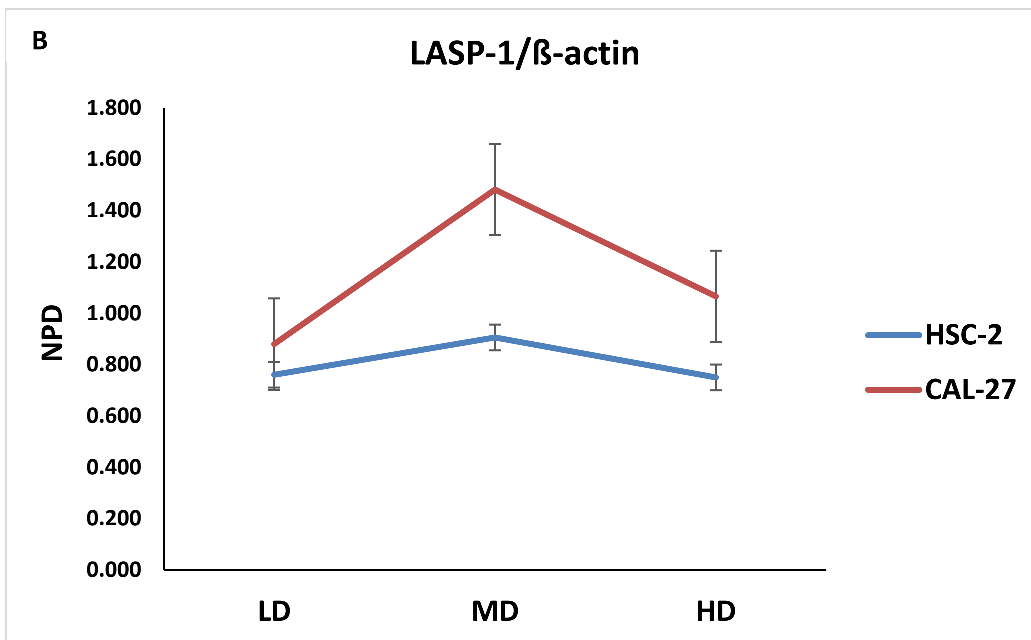


Figure 5. Analysis of LASP1 expression in different cell lines. (**A**). Western blot analysis of LASP1 in SKUT-1, HSC-2, CAL-27, and SK-BR-3 cell lines**;** β-actin was used as a western blotting loading control. Signals were visualized using the BIORAD ChemiDoc™ MP Imaging System. (**B**) Normalized LASP1 expression (NPD) within cell lines HSC-2 and CAL-27 cell lines.

MDA-MB-231 is a highly aggressive, invasive, and poorly differentiated triple-negative breast cancer (TNBC) cell line [20, 21] and is more malignant than SK-BR-3 and MCF-7. In this work, LASP1 results highly expressed in the MDA-MB-231 (regardless of confluence), more than in the SK-BR-3 and MCF-7 cell lines. Thus, it is plausible to speculate that LASP1 correlates to the malignancy of the cancer cell lines. In the resting conditions, LASP1 localizes at the baso-lateral level of cellular membrane, where it regulates the anchorage of cells to ECM, interacting with actin as well as other components of focal adhesions (FAs) [6]. During cell migration and invasion (both crucial events in metastatic process) it localizes in the invasive membrane protrusions such as podosoma, lamellipodia, filopodia and invadopodia [4, 7, 17, 22-24]. Importantly, Endres and co-workers observed that LASP1 improved cell breast and prostate cancer invasion *in vitro,* both directly, acting on cellular projection formation, and indirectly, by promoting the secretion and activation of MMP-1, -2, -3, and -9 in these same invasive cellular protrusions [7].

CAL-27 is a highly malignant and metastatic oral squamous cancer cell line, resistant to drug treatment. Notably, CAL-27 is more metastatic and malignant than HSC-2. Interestingly, the preliminary data of this work demonstrated that the LASP1 resulted more expressed in CAL-27 than in HCS-2 cell line. In particular, the higher expression of LASP1 in CAL-27 compared to its expression within HSC-2 is more evident in the medium density (representing the “exponential phase of cellular growth”). This leads to contemplate, as it has been already speculated for the breast cancer, the idea that the expression of LASP1 also for these two cell lines may be linked to the cell malignancy.

In this experiment was noticed a difference between the LASP1 expression in breast cancer and oral squamous cell carcinoma cell lines (LASP1 in MDA-MB-231 in low density and medium densities is more expressed than in CAL-27 in the densities). But, as was mentioned above, these are preliminary data, thus it is difficult to make some final conclusions or comments, also because the results deal with different types of cancer.

Hypoxia indicates a decrease in the concentration of available oxygen at the tissues level. Hypoxia is able to limit and even arrest the physiological function of organs, tissue and cells [25]. Tumor cells commonly induce hypoxic conditions, caused by the rapid growth of tumor cells and the relatively limited blood supply in tumors [26, 27]. Hypoxia, in its turn, stabilizes HIF-1α, which in form of heterodimeric transcription factor, acts on target genes expression [13]. Interestingly, LASP1 is known to be a target of HIF-1α [15], this aspect allows to suppose that LASP1 may have a crucial role during EMT and consequent cancer aggressiveness.

Treating Human breast cancer cells MDA-MB-231 with $CoCl_2$ permitted to create the hypoxic conditions that are so characteristic for tumor microenvironment. The task of the experiment (using CoCl2 to mimic the hypoxic conditions in breast cancer cells) was to investigate the effect of a hypoxic environment on the cell viability and on the biological behavior of breast cancer MDA-MB-231 cells. The aim of treating MDA-MB-231 cells with 50, 100, and 200 μM $CoCl_2$ for 24h and 48 h (**Fig. 6, Tab. 2**) was to establish a hypoxic model for MDA-MB-231 cells *in vitro*.

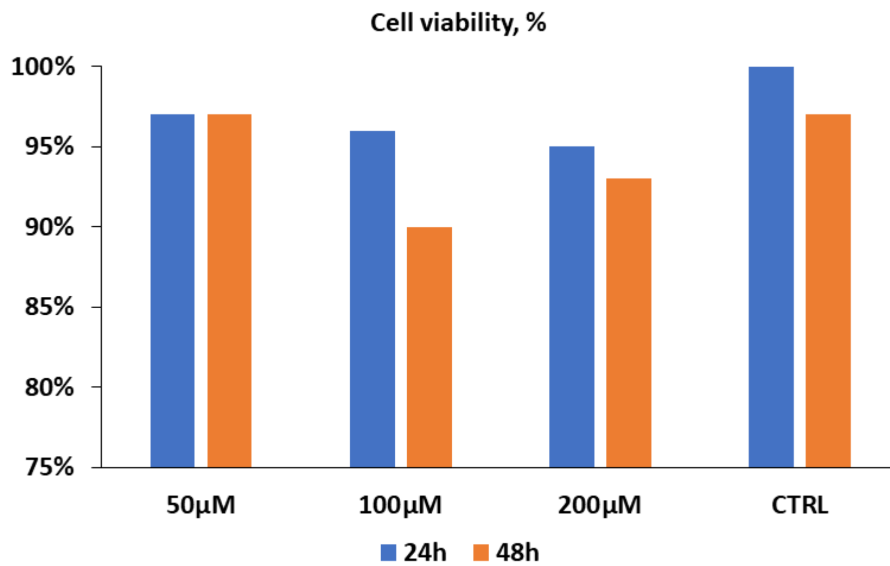


Figure 6. Cellular viability after $CoCl_2$ incubation. The percentage of live cells demonstrated after 24h and 48h incubation with different concentrations (50µM, 100µM, 200µM) of $CoCl_2$. The cell line, used for the cell viability detection, was human breast cancer cells MDA-MB-231.

Table 2. The viability of MDA-MB-231 shown in percentage of live cell after 24h and 48h incubation with different concentrations (50µM, 100µM, 200µM) of $CoCl_2$.

	24h cell viability, %	48h cell viability, %
50 µM $CoCl_2$ concentration	97	97
100µM $CoCl_2$ concentration	96	90
200 µM $CoCl_2$ concentration	95	93
CTRL (no CoCl2 added)	100	97

Part II

Vascular Biology research group Lena Claesson-Welsh, Department of Immunology, Genetics and Pathology (Rudbeck laboratory, Uppsala University, Sweden)

In this laboratory I studied the role of SFKs (especially its member YES) in the dynamics of endothelial junctions in endothelial cells (ECs), and its role in cell migration.

Research Activity

Introduction

Blood and lymphatic vasculature provide all body tissues with vital and essential elements, controls the levels of oxygen and removal of waste products [28]. Notably, the barrier between vascular lumen and around tissues is lined with endothelial cells (ECs) [29].

The EC barrier controls exchange of all contents, arriving with blood [30]. To reach their destination all immune cells, gases, nutrients, hormones and growth factors must go through the EC barrier [31]. Additionally, the opening of junctions between ECs is needed to permit leakage for the passage of cells and larger molecules (>70 kDa) through the barrier [32].

Within lots of diseases (i.e., inflammatory conditions, retinopathies, and cancer) vascular leakage aggravates the disease because of its persistence and debilitation of microenvironment [33].

In the endothelium of established blood vessels, cell–cell junctions connect and coordinate the activities of the individual ECs for the control of vascular permeability, immune cell trafficking, and angiogenesis [34, 35]. The cell–cell junctions in the endothelium are defined as tight junctions (TJs) and adherens junctions (AJs), which are intermixed in ECs [36].

Adherens junctions are composed of the EC-specific vascular endothelial cadherin (VE-cadherin) [37]. VE-cadherin provides homophilic interactions between neighbouring ECs, and is associated with (α)-, (β)- and (γ)-catenins, which in its turn connect to the cytoskeleton [38].

Tight junctions are built up of a range of proteins including claudins, occludins, JAMA and cingulin [39]. Interestingly some of these proteins are EC-specific (i.e., Claudin-5 (Cldn5)), which like VE-cadherin, forms homophilic interactions between ECs [40].

Adherens junctions can disassemble in response to certain stimuli such as inflammatory cytokines (e.g., histamine/bradykinin) and VEGF, by c-Src-mediated VE-cadherin phosphorylation [41] (**Fig. 7**).

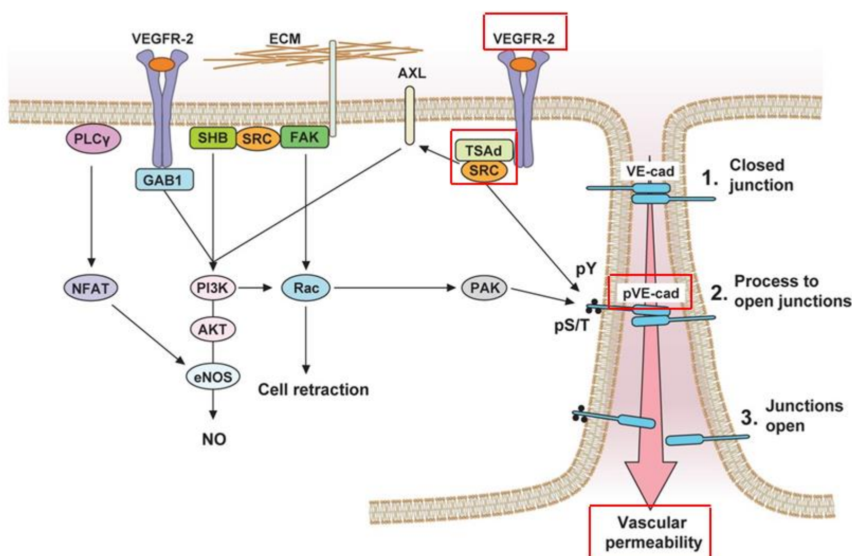


Figure 7. Summary of signalling events downstream of VEGFR2 in regulation of VEGFA-induced vascular permeability. Red rectangles indicate the VEGFR2–TSAd–Src–VE-cadherin pathway. Modified from Claesson-Welsh et al [42].

Thus, the components of adherens junctions have been figured out, but molecules that co-organize and regulate their function remain to be unclear. The composition and regulation of tight junctions has been even less studied. In the CNS, tight junctions are present in all vessels (arteries, capillaries, veins), providing an impermeable barrier that protects the sensitive brain tissue from inflammation, infection and oedema [43].

As mentioned above, the phosphorylation of AJs component VE-cadherin is mediated by Src family kinases' (SFKs) member, c-Src.

SFKs are the group of nonreceptor tyrosine kinases involved in the regulation of various signaling pathways involved in proliferation, survival, migration, angiogenesis, and metastasis (**Fig. 8**).

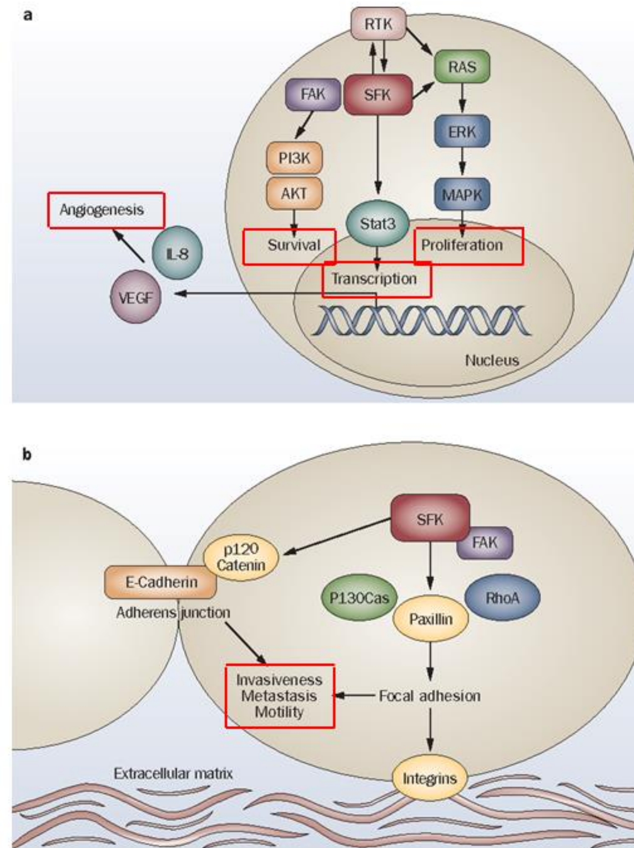


Figure 8. SFK signaling pathways and function. SFKs role in angiogenesis, survival, proliferation, transcription, invasiveness, metastasis, and motility. Modified from Kim et al [44].

SFKs are regulated by different growth factors and antigen receptors and their activation is important for generating appropriate cellular response [45]. There are 10 known members of this family: Src, Frk, Lck, Lyn, Blk, Hck, Fyn, Yrk, Fgr, and Yes [46]. These proteins share four Src homology (SH) domains involved in catalytic activity, protein-protein interaction, and cell membrane binding [47] (**Fig. 9**).

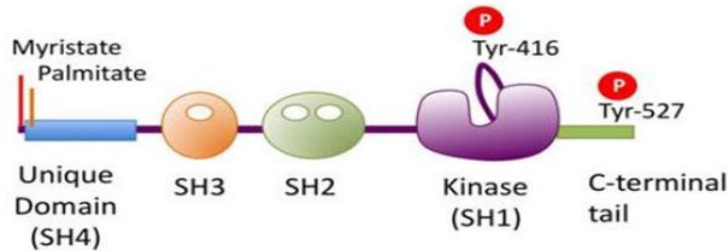


Figure 9. Schematic model of Src family kinases. SFKs conserved domain organization includes a myristoylated N-terminal segment followed by SH3, SH2, and tyrosine kinase domains, and a short C-terminal tail. The two main phosphorylation sites in SFKs are Tyr 416 located on the SH1 domain and Tyr 527 on the regulatory domain near the carboxy terminus. Modified from Okada et al [48].

There are some studies that explain the role of c-Src in the vascular permeability. c-Src activation in a VEGF (Vascular endothelial growth factor)/T cell-specific adapter (TSAd)-dependent manner, induce the activation of vascular permeability [49].

The aim of this study is to figure out the role of another SFK member, YES, in the endothelial junction dynamics.

Methods:

Cell cultures.

Endothelial cell line HUVEC was cultivated in Endothelial Cell GM MV2 medium (Sigma-Aldrich) supplemented with Fetal Calf Serum (FCS) (Sigma-Aldrich) at final concentration 0,05ml/ml (Sigma-Aldrich), Epidermal Growth Factor (recombinant human) (hEGF) 5 ng/ml (Sigma-Aldrich), Hydrocortisone (HC) 0,2µg/ml (Sigma-Aldrich), Vascular Endothelial Growth Factor 165 (recombinant human) (VEGF) 0,5ng/ml (Sigma-Aldrich), Basic Fibroblast Growth Factor (recombinant human) (hbFGF) 10 ng/ml (Sigma-Aldrich), Insulin-like Growth Factor (Long R3 IGF) (R3 IGF) 20ng/ml (Sigma-Aldrich), Ascorbic Acid (AA) 1 µg/ml (Sigma-Aldrich) in a humidified chamber of 5% CO2 at 37°C.

siRNA transfection and starvation assay.

In order to suppress YES and SRC expression, cultured HUVECs were transfected with YES and SRC short interfering (si)RNA (Sigma-Aldrich). An additional non-targeting scrambled siRNA served as a negative control (Sigma-Aldrich). HUVECs were transfected with the siRNAs using LIPOFECTAMINE RNAiMAX (Invitrogen). Then 48h after transfection the cells were incubated for 3 hours with Starvation medium (Endothelial Cell GM MV2 medium without any supplement except 0,2% FCS).

VEGFA stimulation.

After the 3h incubation with Starvation Medium (Endothelial Cell GM MV2 medium without any supplement except 0,2% FCS), cells were stimulated with VEGFA (PeproTech) for 5 and 15 minutes at the final concentration of 50ng/ml.

Cell lysates and protein dosage.

Cells were scrapped and the cell lysates were prepared by solubilization in Lysis buffer (50mM Tris pH 7.5, 10mM $MgCl_2$, 0,5M NaCl, 2% Igepal), containing proteinase and phosphatase inhibitors. The protein content of cell lysates was measured with Bradford method using the SYNERGY HTX multi-mode reader. The cell lysates then were frozen at -80 °C until use.

Western blotting.

The lysates were loaded into NuPAGE™ 2-12% Bis-Tris Gel, 30µg of proteins was loaded in each lane. Proteins were then transferred to PVDF membrane using the XCell II ™ Blot Module (Life technologies). The saturation of membrane was performed with 5% of dry milk diluted in TTBS for normal protein and with 5% BSA diluted in TTBS for phosphorylated proteins for 1 hour at room temperature (RT). Consecutively, the incubation with the specific primary rabbit anti-human pVEGFR2 (phosphorylated Vascular Endothelial Growth Factor Receptor 2) (Y949) antibody (ABCAM), specific primary rabbit anti-human pSTAT1 (Signal transducer and activator of transcription 1) antibody (Cell Signalling), specific primary mouse anti-human VEGFR2 antibody (Santa Cruz), specific primary mouse anti-human STAT1 antibody (Abcam), specific primary mouse anti-human GAPDH antibody (Abcam), specific primary mouse anti-human YES antibody (BD bioscience) diluted in 5% dry milk for normal proteins (1:1000) and in 5% BSA for phosphorylated proteins (1:1000) was done and was left overnight at +4°C. The next day, after 3 washes in TBS-T, the membrane was incubated with a ECL™ Anti-rabbit IgG, Horseradish Peroxidase linked whole antibody (from donkey) (GE Healthcare Life Sciences) and ECL ™ Anti-mouse IgG, Horseradish Peroxidase linked whole antibody (from sheep) (GE Healthcare Life Sciences) respectively, diluted in 5% BSA for phosphorylated proteins and in 5% dry milk for normal proteins (1:10000) for 1h at RT. Signals were visualized using the BIORAD ChemiDoc™ MP Imaging System (**Fig. 10**). The analysis of proteins expression was done with Image Lab software (**Fig. 11, 12**).

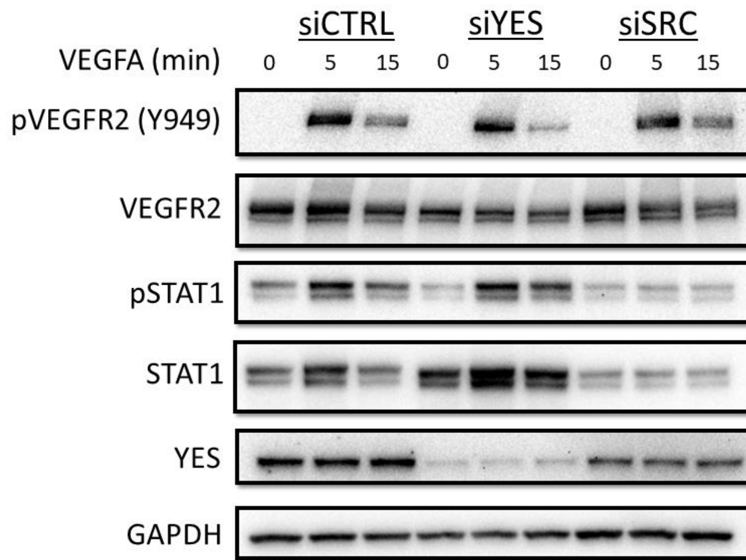


Figure 10. Western Blotting analysis of VEGF/VEGFR2 signal pathway. The analysis of proteins expression was done within siCTRL, siYES, siSCR HUVECs treated (for 5 min and 15 min) or not with VEGFA (50 ng/ml). Membranes treated with specific primary antibodies (VEGFR2 and pVEGFR; STAT1 and pSTAT1; YES) and with a peroxidase-conjugated secondary antibodies. The analysis of proteins expression was done within siCTRL, siYES, siSCR HUVECs treated (for 5 min and 15 min) or not with VEGFA (50 ng/ml).

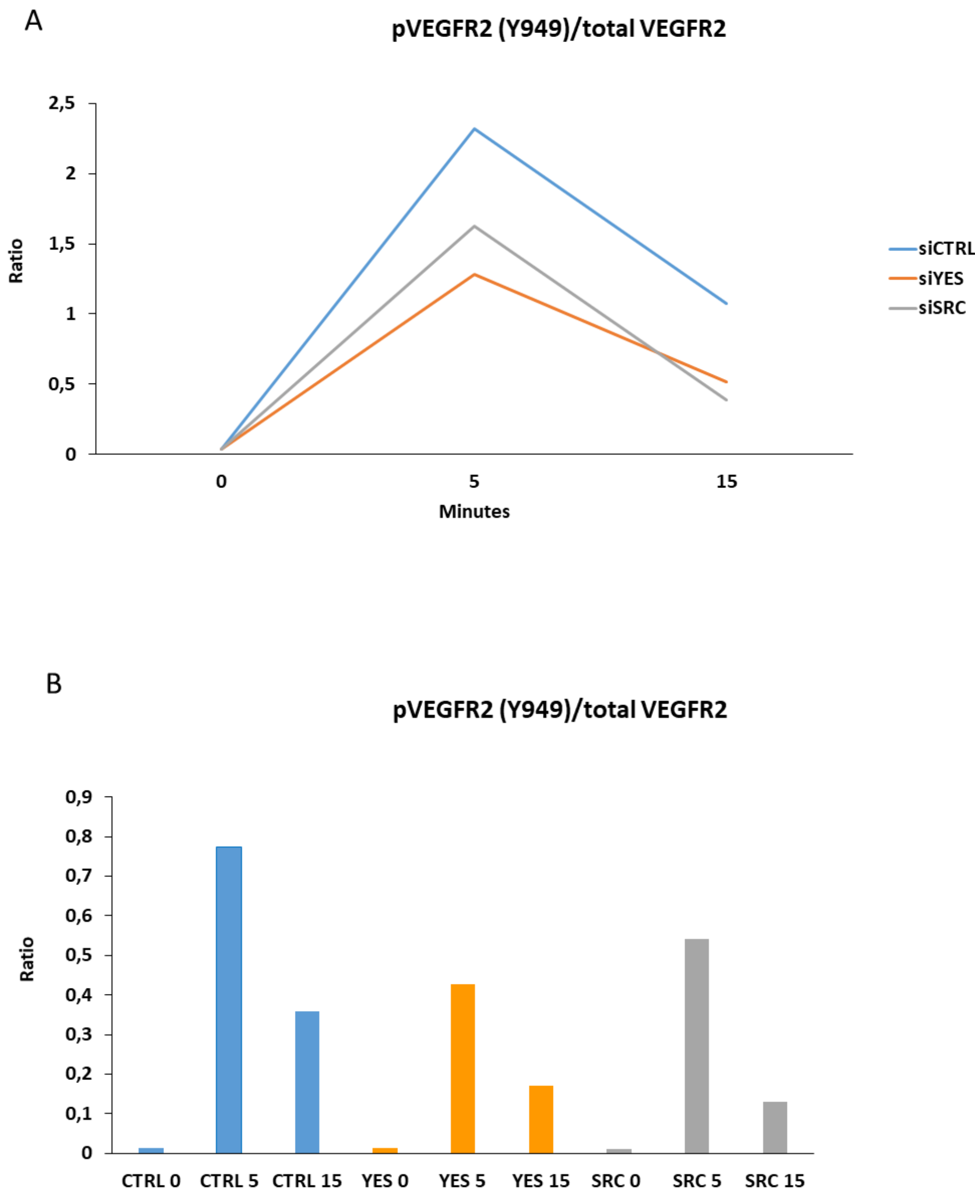


Figure 11A, 11B. Normalized pVEGFR2 expression. Expression levels of all proteins blotted for was analyzed by Image Lab software. Expression level of pVEGFR2 (Y949) initially normalized to GAPDH expression, after that VEGFR2 normalization to GAPDH was done, and as a final step the ratio of normalized pVEGFR2 to normalized VEGFR2 was found. The analysis of proteins expression was done within siCTRL, siYES, siSCR HUVECs treated (for 5 min and 15 min) or not with VEGFA (50 ng/ml). Experiments were performed at least three independent times.

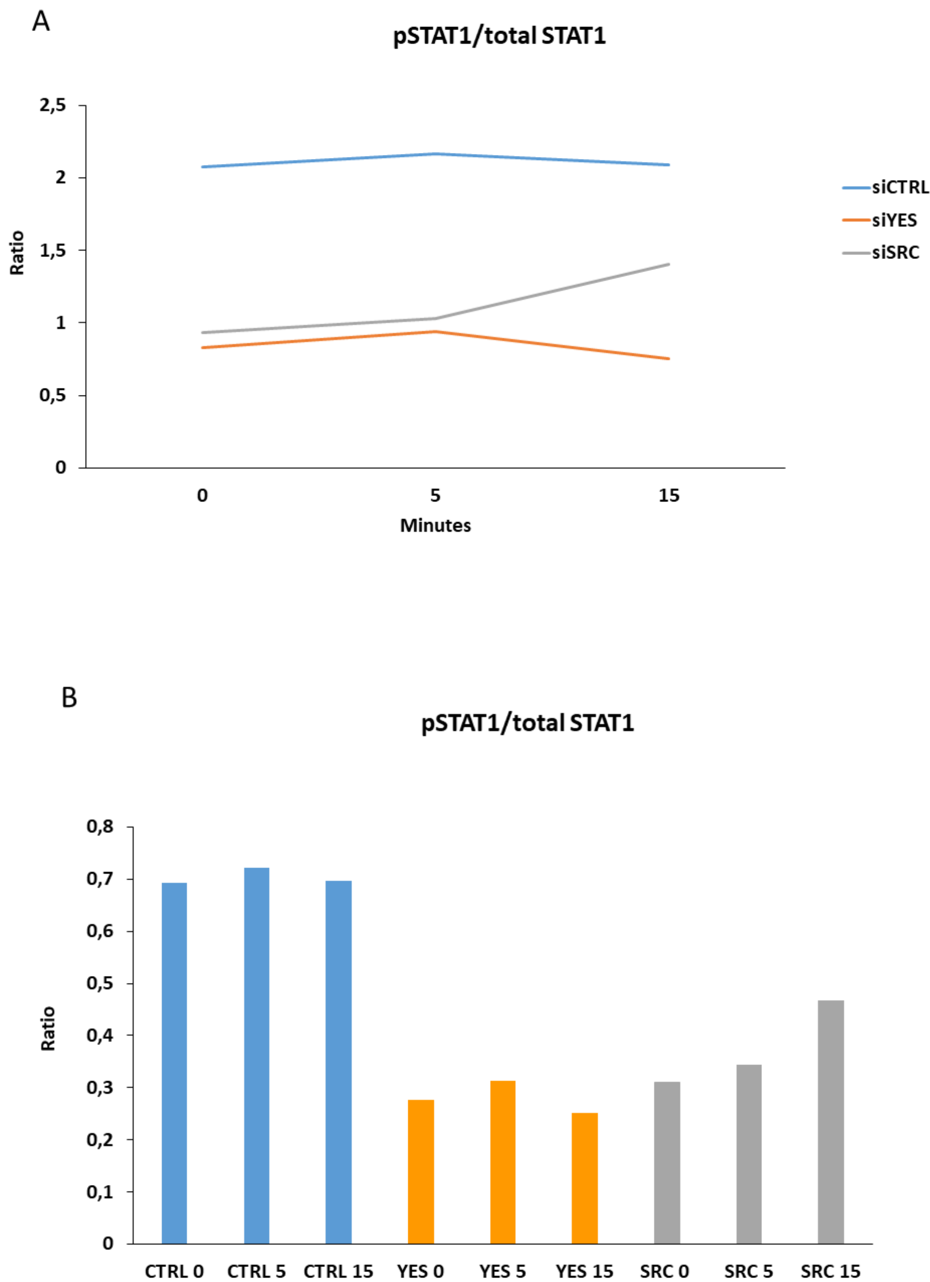


Figure 12A, 12B. Normalized pSTAT1 expression. Expression levels of all proteins blotted for was analyzed by Image Lab. Expression level of pSTAT1 initially normalized to GAPDH expression, after that STAT1 normalization to GAPDH was done, and as a final step the ratio of normalized pSTAT1 to normalized STAT1 was found. The analysis of proteins expression was done within siCTRL, siYES, siSCR HUVECs treated (for 5 min and 15 min) or not with VEGFA (50 ng/ml). Experiments were performed at least three independent times.

***Immunofluorescence staining analysis*.**

The cells were seeded into the 6-well plate upon cover slips (Menzel-Gläser), and after transfection, incubation with Starvation medium and stimulation with VEGFA for 5 min and 15 min, cells were fixed with 2 ml per well of 4% paraformaldehyde for 10 min at RT, washed with PBS-T (Triton 0,05%) three times (5 min/time) and permeabilized with a mixture of 3% BSA and 0,3% Triton X-100. The cells were immunofluorescently stained with pPaxillin (pPAX) (Invitrogen) antibody, which was diluted 1:500 in 1% BSA and 0,1% Triton Blocking buffer, overnight at 4 °C. The cells were then washed with PBS-T three times (5 min/time) and incubated with donkey anti-rabbit IgG (H+L) ReadyProbes™ Secondary Antibody diluted in 1:500 in 1% BSA and 0,1% Triton Blocking buffer in the dark at room temperature for 1 h. Then cells were washed with PBS three times (5 min/time). The cover slips were mounted with DAPI Fluoromount-G (SouthernBiotech) onto the Menzel-Gläser Superfrost Plus (Thermo Scientific). Cells were examined by Leica SP8 LIGHTNING Confocal Microscope (**Fig. 13**).

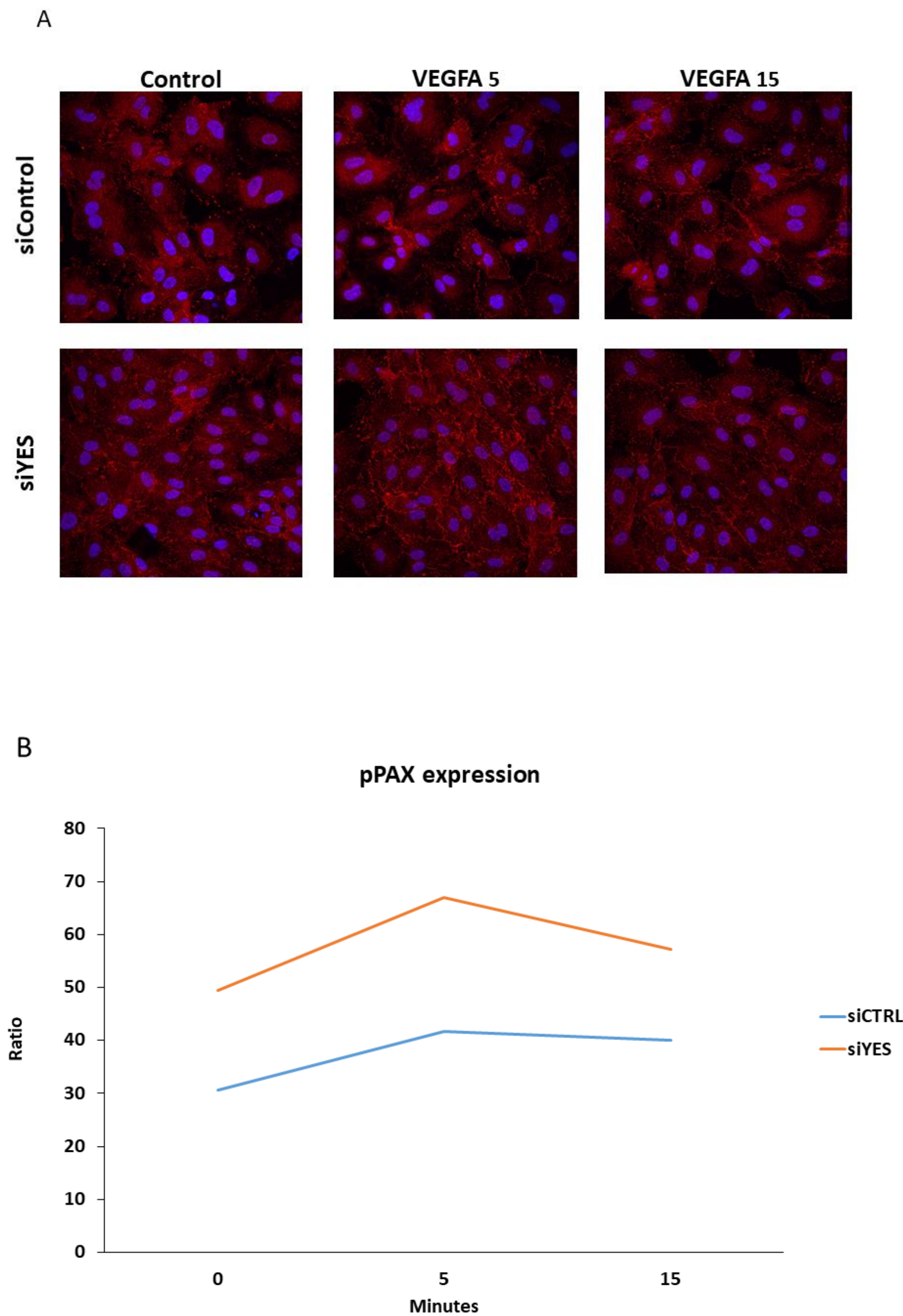


Figure 13. pPAX immunostaining in HUVECs. (**A**) pPAX immunostaining (red) on siCTRL and siYES HUVECs treated (for 5 min and 15 min) or not with VEGFA (50 ng/ml). DAPI (blue) staining shows nuclei. (**B**) pPAX-positive staining normalized to cell quantity for each condition.

Scratch assay and live imaging.

Several hours before live imaging SiR actin dye (Cytoskeleton, Inc.) was added to the wells previously silueted in Endothelial Cell GM MV2 medium. Right before live imaging the scratch assay was performed with a tip in both studied conditions: HUVEC siCTRL and HUVEC siYES. After that the the medium within the wells was changed to the fresh Endothelial Cell GM MV2 medium. Live imaging was performed for 15 hours with Leica SP8 LIGHTNING Confocal Microscope (**Fig. 14**).

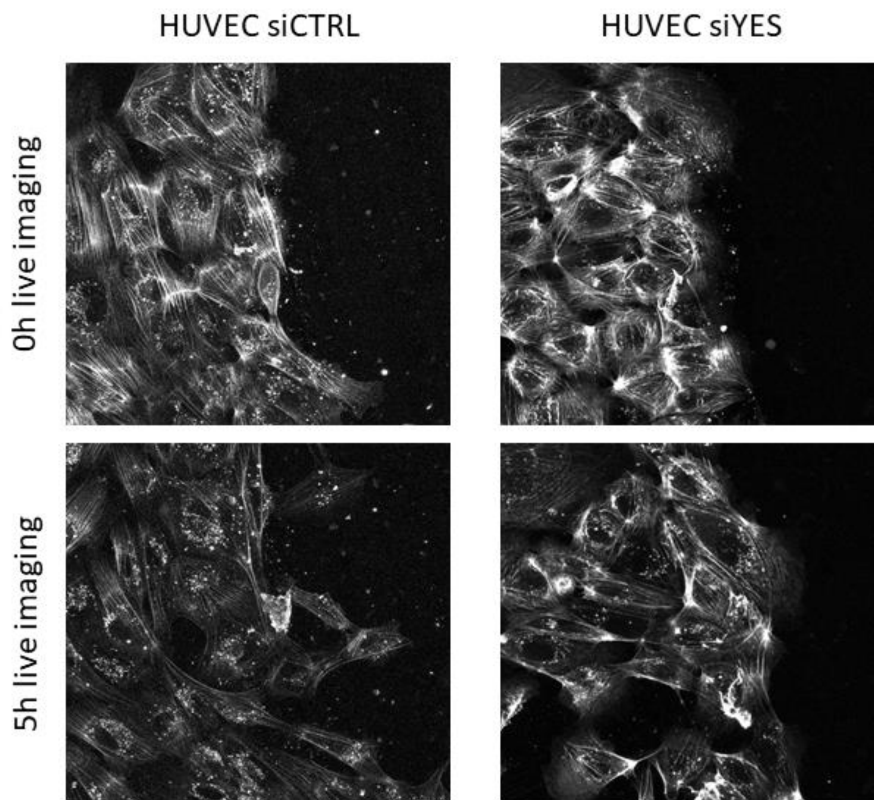


Figure 14. Live imaging of HUVEC siCTRL and HUVEC siYES. Imaging was accomplished for 5 hours after using SiR actin dye and after performing the scratch assay.

Preliminary results and discussion

Endothelial cells maintain homeostasis by regulating the passage of cells, fluid, and protein from the vascular space to the interstitial space [50]. Physiologic responses to trauma, infection, and tumor growth involve the production of cytokines and growth factors that bind their cognate receptors on endothelial cells. Such receptor binding results in the tyrosine phosphorylation of numerous molecules that effect changes in vascular permeability, including SFKs [51].

SFKs are signalling enzymes that have been recognized to regulate critical cellular processes such as proliferation, survival, migration, and metastasis. [47]. Studies of signalling pathways leading to the activation of Src and its closely related homologs in SFKs have been central toward understanding normal growth-regulatory processes, including proliferation, apoptosis, cell cycle control, angiogenesis, cell-cell adhesion, and communication [52].

Src associates with adherens junctions by interacting with VE-cadherin [53]. Upon association in protein complexes, Src can phosphorylate VE-cadherin [54]. VEGF-A is produced under physiologic conditions by numerous cells, including tumor cells, and signals through receptor tyrosine kinases via autocrine and paracrine mechanisms [55]. Upon VEGF-A binding to its cognate receptors (VEGFR1 and VEGFR2 in vascular ECs), the intrinsic tyrosine kinase activity of these receptors is activated, leading to trans-phosphorylation and direct interaction with many SH-2-containing signalling molecules, including Src [56]. Once bound to VEGFR1 or VEGFR2, Src undergoes a conformational change leading to its activation and the subsequent phosphorylation of VE-cadherin [57]. The study of molecules that co-organize and regulate the functions of endothelial junctions is very important for better understanding of their dynamics in normal and pathological conditions.

Analysing the expression levels of phosphorylated STAT1, it is noticeable, that siRNA-mediated YES knock-down (siYES) HUVECs have the lowest expression levels of pSTAT1 (**Fig. 10,12**). The difference between siRNA-mediated Src knock-down (siSRC) HUVECs pSTAT1 levels and

HUVECs transfected with a non-targeting control siRNA (siCTRL HUVECs) is observable. Data suggest that SFKs are involved in activation of STAT1.

Paxillin, being one of the first adaptor molecules identified within adhesions, is a canonical adapter protein containing four LIM domains (double-zinc finger domains that regulate protein interactions) of which LIM2 and LIM3 are important for targeting paxillin to adhesions [58]. Paxillin contains many phosphorylation sites that act as substrates for kinases and phosphatases that regulate cell migration and adhesion dynamics [59]. Paxillin tyrosine phosphorylation is associated with the focal adhesion formation during cell migration [59]. Analysing immunostaining experiment results, I compare the quantity of pPAX within siYES HUVECs and siCTRL HUVECs (**Fig. 13**). It is noticeable that there is more pPAX within siYES HUVECs than in siCTRL cells. It is also plausible, that in siYES cells pPAX is more distributed in the focal adhesion sites. The highest level of PAX phosphorylation is observed in siYES cells treated for 5 min with VEGFA. The increased adhesion site size phosphoryation of paxillin might provoke the disassembly of adhesion sites what in its turn effect cell motility.

By analysing the results of live imaging performed within HUVEC siCTRL and HUVEC siYES it becomes evident the differences between these 2 conditions. The movements of HUVEC siYES were less orchestrated, and the cells were more tended to lose and break adherens junctions. In the contrast, the movements of HUVEC siCTRL were more organised, more structured, and the adherens junctions were more stable in comparison to HUVEC siYES (**Fig. 14**).

Better understanding of the mechanisms of SFK influence on cell-cell connection remodelling has an enormous interest in the therapeutic potential of SFK moderation treatment within inflammation-related illnesses and neoplasms.

Part 3

The role of natural compound in chemioprevention and chemiotherapy

Another area of my research focus were natural compounds in chemoprevention/chemotherapy. To structure and highlight all studied information I did literature overview of two natural compounds: piperine and ginger.

Since cancer is one of the most common and incurable diseases in humans, several pharmacological approaches have been developed (radiotherapy, chemotherapy, surgical intervention, target therapy) for the treatment of tumors and related metastasis. However, many counterproductive effects have been found including multidrug resistance [60]. It was evidenced that a healthy diet coupled with the consumption of specific phytochemical compounds may play a protective role against tumor onset [61-64]. According to this idea, it has been recently introduced the concept of “chemoprevention”, a term that refers to natural agents with the capability to hinder the malignant transformation and dissemination with negligible side effects [61]. The chemopreventive molecular mechanisms of piperine and ginger derivatives include cell cycle arrest, induction of cancer cell death, misbalancing of redox homeostasis, inhibition of cell proliferation, angiogenesis, migration, and dissemination of cancer cells in different cancer types (**Fig. 15**).

The published reviews of chemopreventive/chemotherapeutic effects of piperine and ginger are presented below.

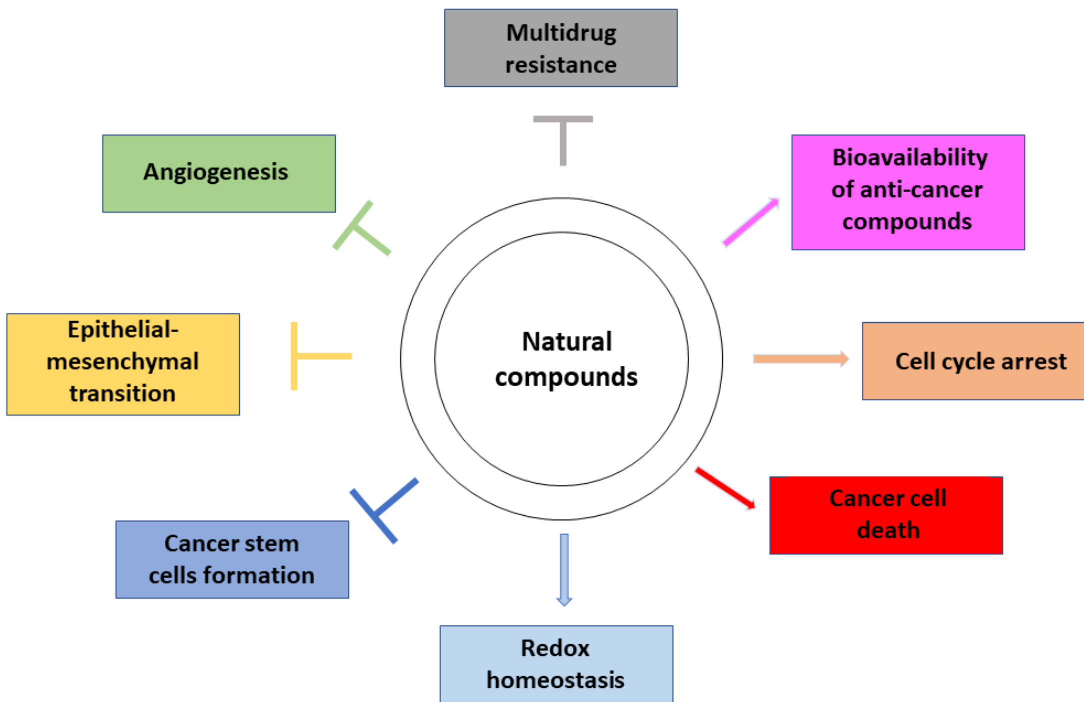


Figure 15. Schematic chemopreventive mechanisms of natural compounds. Natural compounds induce cell cycle arrest, cancer cell death and misbalancing of redox homeostasis. Furthermore, these compounds inhibit cancer stem cell formation, angiogenesis, epithelial-mesenchymal transition and, in parallel, decrease multidrug resistance.

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REVIEW

Piperine: role in prevention and progression of cancer

Mariia Zadorozhna[1] · Tiziana Tataranni[2] · Domenica Mangieri[1]

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Abstract

Cancer is among the leading causes of death worldwide. Several pharmacological protocols have been developed in order to block tumor progression often showing partial efficacy and severe counterproductive effects. It is now conceived that a healthy lifestyle coupled with the consumption of certain phytochemicals can play a protective role against tumor development and progression. According to this vision, it has been introduced the concept of "chemoprevention". This term refers to natural agents with the capability to interfere with the tumorigenesis and metastasis, or at least, attenuate the cancer-related symptoms. Piperine (1-Piperoylpiperidine), a main extract of *Piper longum* and *Piper nigrum*, is an alkaloid with a long history of medicinal use. In fact, it exhibits a variety of biochemical and pharmaceutical properties, including chemopreventive activities without significant cytotoxic effects on normal cells, at least at doses < of 250 μg/ml. The aim of this review is to discuss the relevant molecular and cellular mechanisms underlying the chemopreventive action of this natural alkaloid.

Keywords Piperine · Chemoprevention · Natural compounds · Cancer therapy

Abbreviations

AKT	Protein kinase B
CAM	Chick chorioallantoic membrane
CDKs	Cyclin-dependent kinases
CHOP	C/EBP homologous protein
CKIs	Cyclin-dependent inhibitors
CYPs	Cytochromes-P450
CSCs	Cancer stem cells
DNA	Desossiribonucleic acid
DMEs	Drugs metabolizing enzymes
DENA	Diethylnitrosamine
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK1/2	Extracellular-regulated kinase 1/2
FAs	Focal adhesions
GPX	Glutathione peroxidase
GR	Glutathione reductase
GRP78	Glucose-regulated protein 78
HUVECs	Human umbilical vein endothelial cells
HER2	Epidermal growth factor receptor-2
IGF	Insulin-like growth factor
IRE1a	Inositol-requiring enzyme-1-a
JNK	Jun N-terminal kinase
LC3II	Phosphatidylethanolamine conjugate 3II
MMPs	Matrix metalloproteinases
mTOR	Mammalian target of rapamycin
mTORCs	Mammalian target of rapamycin complexes
MAPK	Mitogen-activated protein kinase
NF-Kb	Nuclear factor-κB
Nox	Nicotinamide adenine dinucleotide phosphate oxidases
PARP	Poly-ADP ribose polymerase
PMA	Phorbol-12-myristate-13-acetate
PI3 K	Phosphoinositide-3 kinase
PKCα	Phospho-kinase C alpha
P-gp	P-glycoprotein
ROS	Reactive oxygen species

Mariia Zadorozhna and Tiziana Tataranni have contributed equally to the present review.

✉ Domenica Mangieri
domenica.mangieri@unifg.it
http://www.medicina.unifg.it

Mariia Zadorozhna
mariia.zadorozhna@unifg.it

Tiziana Tataranni
tiziana.tataranni@crob.it

[1] Department of Medical and Surgical Sciences, University of Foggia, Via Pinto 1, 71122 Foggia, Italy

[2] Laboratory of Pre-Clinical and Translational Research, IRCCS-CROB, Referral Cancer Center of Basilicata, Rionero in Vulture, PZ, Italy

STAT-3	Signal transducer and activator of transcription-3
TGF-β	Trasformin growth factor-beta
TQ	Thymoquinone
UV	Ultraviolet
UDP-GDH	Uridine diphosphate -glucose dehydrogenase
UDP-GT	Uridine diphosphate-glucoronyl transferase
VEGF	Vascular endothelial growth factor/receptors
VEGFRs	VEGF receptors
XO	Xanthine oxidase

Introduction

Cancer is one of the most common and incurable diseases in human in spite of improved approaches to the detection and treatment of this pathology. Several pharmacological approaches have been developed (radiotherapy, chemotherapy, surgical intervention, target therapy) for the treatment of tumors and related metastasis, however many counterproductive effects have been found including multidrug resistance [1]. Genetic, environmental and lifestyle factors can contribute to cancer risk. In fact, as emerged from experimental studies, several elements including unhealthy diet, cigarette smoking, alcohol consumption, environmental carcinogens, ultraviolet (UV) exposure, stress, physical inactivity as well as hormonal factors, strictly correlate to cancer onset and dissemination [2–4]. By contrast, a healthy diet coupled with the consumption of specific phytochemical compounds may play a protective role against tumor onset [5–8]. According to this idea, it has been recently introduced the concept of “chemoprevention”, a term that refers to natural agents with the capability to hinder the malignant transformation and dissemination with negligible side effects [5]. Piperine is one of the most widespread dietary alkaloid (Fig. 1) principally found in the fruits and roots of Piper nigrum (black pepper) and Piper longum (long pepper) [9]. This compound is well known for its anti-inflammatory, immunosuppressive and antibiotics proprieties [9]. Additional studies attribute to piperine also chemopreventive/anti-cancer actions [10–15]. In this regard, the main cellular biological effects of piperine in vitro at specific doses (75–200 μM) and time of incubation (24–48 h) seem to include: inhibition of cellular proliferation and migration, arrest of cell cycle, induction of cancer cells death, alterations in redox homeostasis, modulation of angiogenesis and degradation of extracellular matrix (ECM) as detailed below (Fig. 2c, Table 1). Remarkably, it was also found that piperine may enhance tumour’s susceptibility to current anti-neoplastic drugs [1, 16, 17]. Interestingly, piperine shows an anti-tumor action also in animal models [8, 9]. Here we review the current knowledge about pharmaceutical properties of piperine emphasizing the main relevant molecular and cellular mechanisms underlying its chemopreventive action (Fig. 3).

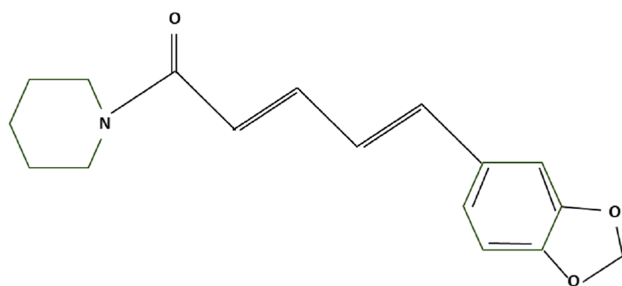

Fig. 1 Chemical structure of piperine (1-[5-(1,3-benzodioxol-5- yl)-1-oxo-2,4-pentadienyl]). Piperine is a hydrophobic alkaloid containing a heterocyclic ring structure and a basic nitrogen atom located inside the ring structure

Piperine arrests cell cycle and cancer growth

Piperine is able to modulate cell cycle progression as a part of their chemopreventive mechanism [18] (Fig. 2a). Cell cycle is crucial to maintain cell proliferation and tissues integrity; it is, therefore, carefully regulated in well-defined checkpoints by specific proteins and kinases including cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs) [19]. Deregulation of cell cycle and/or its arrest is often responsible of severe pathologies, including cancer [19, 20]. As mentioned above, piperine shows the ability to control the relevant checkpoints of cell cycle in tumor, contrasting with its progression [21]. For example, piperine results cytotoxic for human and murine melanoma cells (SKMEL-28 and B16F0, respectively) in which it causes arrest of cell cycle at G1 phase by downregulating the level of cyclin D1 and the activation of CDK inhibitor-1 (p21/WAF1) [22]. In these same circumstances, it was observed that the alkaloid disrupts reactive oxygen species (ROS) homeostasis that, in turn, causes DNA, lipids and proteins damages [22]. Similar results were observed in vitro in human colon cancer, in which piperine arrests cell cycle at G1 phase downregulating D1 and D2 cyclins and their partners CKD4 and CDK6 [23]. In this instance, piperine facilitated a reduction in phosphorylation of the retinoblastoma (Rb) protein and an up-regulation of p21/WAF1 and p27/KIP1 expression [23]. Comparable outcomes were reported in human prostate cancer cell lines, both in androgen dependent PC3 cells and androgen independent cells like LNCaP and DU145 lines, in which piperine arrests cell cycle at G0/G1 phase interfering with the expression of the CDK inhibitors p21 and p27 [24]. Other investigations showed that 1-Piperoylpiperidine is also able to block cell cycle at G2/M phase in some tumor models, including human osteosarcoma cells and in murine breast cancer (4T1

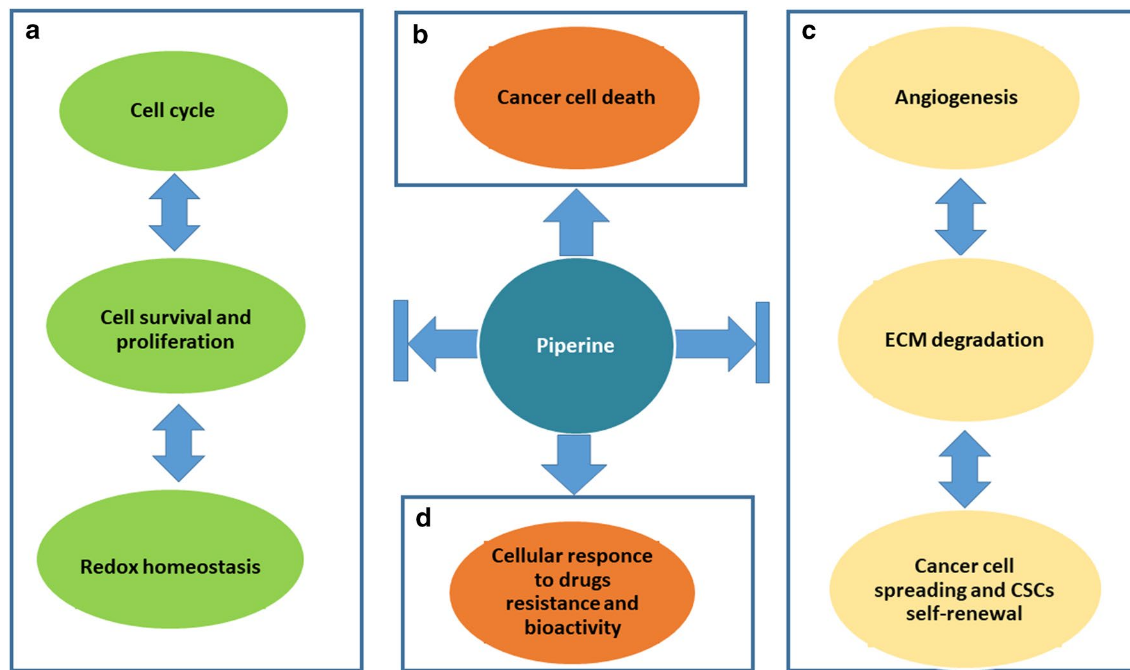

Fig. 2 Schematic anti-cancer action of piperine. **a** The alkaloid reduces cancer cell proliferation, interferes with cell cycle, cause an imbalance in cellular redox homeostasis, **b** and induces cell death. **c** Additionally, piperine inhibits angiogenesis, ECM degradation and thereby impedes the spreading of tumor cells and inhibits CSCs self-renewal. **d** Furthermore, Piperine enhances the bioactivity of chemical and natural drugs

Table 1 Effects of piperine in different types of cancers

Tumor entity	Functions of piperine	References
Breast cancer	Arrest of cell cycle at different checkpoint in relation to cancer cell lines; unbalance of ROS homeostasis and induction of apoptosis	[26, 49, 57]
Prostate cancer	Interruption of cell cycle at G0/G1 checkpoint through the inhibitory activity of CDK; down-regulation of p21 and p27; induction of apoptosis by means of caspase-3 and PARP cleavage	[24, 43, 83]
Osteosarcoma	Blockage of the cell cycle at G2/M phase by down-regulating cyclin B1 and by increasing the phosphorylation level CDK1and Chk2	[25]
Melanoma	Arrest of cell cycle at G1 phase through downregulation of cyclin D1, activation of CDK (p21/WAF1) and unbalance of ROS homeostasis	[22]
Colon carcinoma	Downregulation of D1 and D2 cyclins and their partner CKD4 and CDK6 with consequent arrest of cell cycle at G1 phase; mTORC1 inhibitory activity promoting cancer cell death	[23, 41]
Fibrosarcoma	Inhibition of PKCα and ERK1/2 phosphorylation and reduction of NF-κB and AP-1 nuclear translocation, so leading to down-regulation of MMP-9 expression	[56]
Ovarian cancer	Activation of intrinsic pathway of apoptosis after the release of mitochondrial cytochrome c to cytosol, the activation of caspase-3 and -9, the PARP cleavage and the inactivation of p38/MAPK and JNK	[44]
Lung cancer	Reduction of oxidative stress mediated by mitochondrial activities and enhancement of both enzymatic and non-enzymatic defence systems	[76, 77]
Rectal adenocarcinoma	Stimulation of ROS generation leading to dissipation of mitochondrial membrane potential, caspases cascade and apoptosis	[81]
Oral squamous carcinoma	Mediation of mitochondrial pathway of apoptosis.	[21]
Hepatocellular carcinoma	Interaction with CYP1A1 enzyme, mediating deficiency of benzo(a)pyrene (BP) metabolism and consequently abolishing cancer aggressiveness	[80]
Breast cancer stem cells	Inhibition of Wnt/beta-catenin signalling pathway; repression of mammosphere formation	[95, 97]

Molecular action of piperine in different kind of cancer

ROS reactive oxygen species, *CDK* cyclin-dependent kinases, *p21* cyclin-dependent kinase inhibitor/p21, *p27* cyclin-dependent kinase inhibitor 1B/p27, *PARP* poly-ADP ribose polymerase, *CD1* cyclin D1, *CDKs* cyclin-dependent kinases, *mTORC1* mammalian target of rapamycin complex-1, *PKCα* phospho-kinase C-alpha, *ERK1/2* extracellular-regulated kinase 1/2, *NF-κB* nuclear factor-κB, *AP-1* activator protein 1, *MMP-9* matrix metalloprotease-9, *MAPK* mitogen-activated protein kinase, *JNK* Jun N-terminal kinase, *CYP1A* cytochrome-P450 1A

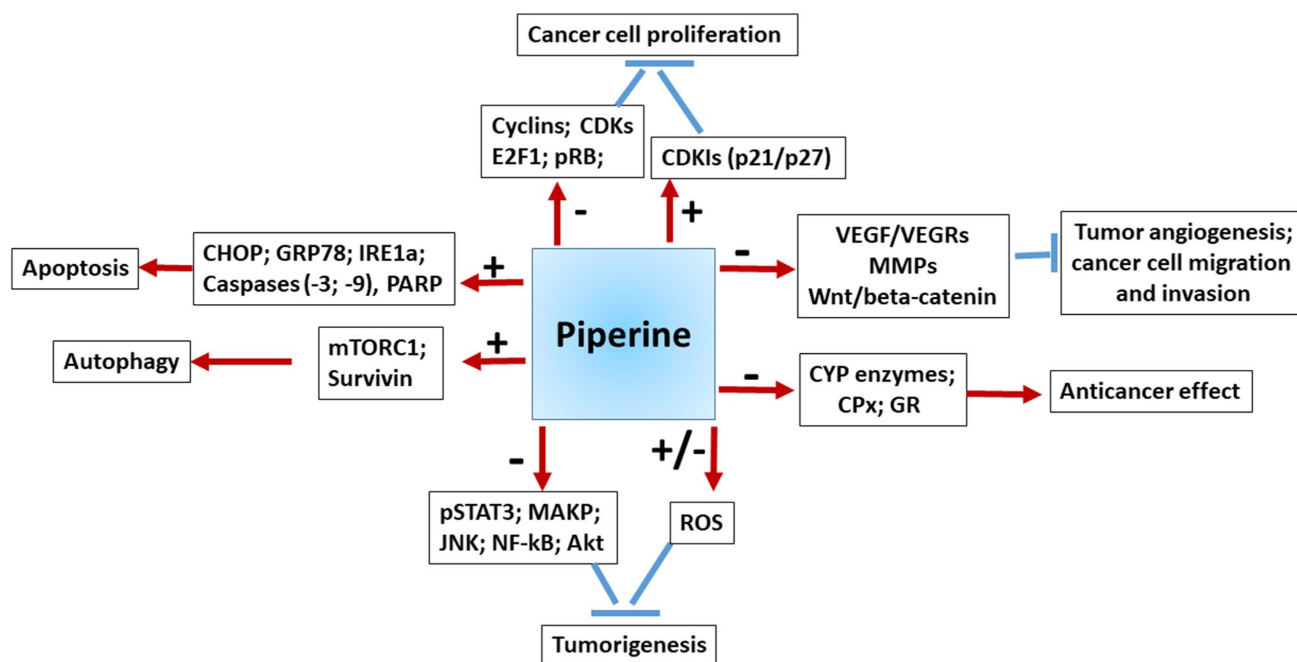

Fig. 3 A schematic diagram showing main molecular targets of Piperine. The alkaloid interferes with cancer proliferation by inhibiting the expression of cyclins, CDK and other molecular regulators of cell cycle (e.g. E2F1, pRb). Piperine exerts anti-migratory, anti-invasive and anti-metastatic effects by blocking the VEGF/VEGFRs signal pathway and MMPs. Piperine rises in CYP 450s and glutathione-metabolizing enzymes (GPx, GR), which indicated anti-cancer effect. Moreover, as anticancer compound, by down-regulating oncogenic factors (e.g. JNK, pSTAT-3, Akt) and by interfering with ROS signal pathway, piperine is able to abolish tumorigenesis. Piperine selectively induces tumor cell death (apoptosis and autophagy) by acting the expression and activities of related proteins and signals

cells), both in vitro and in vivo, by down-regulating cyclin B1 and by increasing the phosphorylation level of cyclin-dependent kinase-1 (CDK1) and kinase 2 (Chk2) [25, 26]. Additionally, Jain and co-workers have shown that piperine increased the generation of intracellular ROS level in HeLa and MCF-7, which arrest cell cycle at G2/M phase with a consequent reduction in cell viability [27]. When human triple-negative breast cancer lines (TNBC: MDA-MB-231 and MDA-MB468) are treated with piperine, the cell cycle is blocked at G1 phase through inactivation of G1-associated cyclin D3 and CDK4 connected with down-regulation of the transcription factor E2F-1 (utilized by growth factors in promoting entry into cell cycle). In the same cancer model, it was also reported that the piperine affects G2-associated cyclins B, CDK1 and Cdc25C [28]. In translational terms, intratumoral administration of piperine (0.2 mg/kg) into fully developed tumours (approximately 100 mm^3 in volume) every other day for a total of three injections, strongly inhibited the growth of TNBC (MDAMB-468 cells) xenografts in NOD/SCID mice [28].

It seems evident that the sum of all these effects reduces the survival of neoplastic cells and supports the chemopreventive action of piperine.

Piperine induces autophagy and/or apoptosis in cancer cells

Autophagy is a self-degradative process important for balancing sources of energy in the embryo development and in response to several causes of cellular stress. This phenomenon is characterized by a cascade of events including degradation of cytoplasmic proteins or entire organelles [29]. Autophagic process is often associate with a variety of sources of stress, comprising deprivation of growth factors and nutrients, inhibition of receptor tyrosine kinases/Akt/mammalian target of rapamycin (mTOR) signaling, inhibition of proteasome, endoplasmic reticulum (ER)-derived stress and unbalanced of ROS homeostasis [30–32]. Conventionally, autophagy is recognised as an event able to overstep all these stresses, allowing cell survival [33]. Remarkably, this phenomenon was initially proposed as a suppressor of tumorigenesis [34] while more recent observations have demonstrated that there is a link among autophagy, tumorigenesis and cancer invasion [35]. Actually, the correlation between autophagy and cancer is controversial; in this context, it seems that autophagy could function as tumor activator, directly affecting the dynamic assembly/disassembly of cell–matrix focal adhesions (FAs), which are essential

for an efficient migration and invasion of cells [36], and/or as tumor suppressor, provoking a programmed cell death of type II [37]. In cancer, as in health, autophagy depends on several prerequisites of stress including microenvironment conditions (hypoxia, nutrients availability, angiogenesis, etc.) [38]. Concerning the pro-death role of autophagy in tumor, in addition to current chemical drugs, some phytochemicals and natural compounds have been identified as efficient agents in inducing cell-death through autophagy, including curcumin and piperine [24, 39]. As demonstrated by Ouyang et al, piperine is responsible of cell cycle arrest and programmed cell death of type II (autophagy) in human prostate cancer (LNCaP and PC3 cell lines), documented by an increase in cellular level of phosphatidylethanolamine conjugate 3II (LC3II/ATG8), a crucial marker of this phenomenon [24]. A well-known pathway controlling cell survival and deregulated autophagy in several human diseases, including cancer, is mTOR complexes signaling, interacting with several proteins to form two distinct complexes named mTOR complex 1 (mTORC1) and 2 (mTORC2) [40]. mTORC1 signaling pathway through a variety of environmental cues controls cell cycle and growth, homeostasis and metabolism [40]. In a recent paper, it was reported that in colon carcinoma, piperine exhibited mTORC1 inhibitory activity promoting cancer cell death in vitro [41]. Based on these observations, it appears evident that autophagy and apoptosis can be envisioned as two phenomena strictly interconnected that could serve to abrogate cancer. The process of apoptosis is generally characterized by well-defined cell morphological features and energy-dependent biochemical mechanisms. Apoptosis is usually executed through two major ways: mitochondria-mediated intrinsic pathway and death receptor-mediated extrinsic route [42]. At the molecular level, piperine can induce both intrinsic and extrinsic signaling of this phenomenon (Fig. 2b). Yaffe et al. demonstrated that piperine caused EE-stress dependent in colon cancer cells (HT-29) activating correlated pro-apoptotic signals such as upregulation of C/EBP homologous protein (CHOP), Glucose-regulated protein 78 (GRP78), Inositol-requiring enzyme-1(IRE1a) and NH2-terminal kinase (JNK) [23]. The overexpression of these pro-apoptotic proteins were accompanied by reduction in Akt phosphorylation and survivin expression, both implicated in cell survival mechanisms [23]. Activation of caspases and consequent apoptosis were also observed in murine and human breast cancer cells following piperine treatment [26]. Furthermore, in androgen-dependent and androgen-independent prostate cancer cell lines, (PC3 and LNCaP, DU145 respectively) the alkaloid induced programmed cell death in vitro [24]. These results were confirmed by activation of caspase-3 that, in turn, cleaved PARP (in its cleaved form, PARP is another positive regulators of apoptosis) [24]. Piperine also caused an inhibition of both phosphorylation of STAT-3 and nuclear translocation of the transcription factor NF-kB [43]. The anti-proliferative/pro-death effect of piperine in prostate cancer was also confirmed in in vivo studies. In fact, tumour growth was substantially reduced following piperine treatment in nude mice subcutaneously implanted with LNCaP and DU-145 cells. In detail, results showed that piperine treatment (100 mg/kg body weight) via intraperitoneal injection against LNCaP xenotransplants resulted in a 72% reduction in tumour size as compared to the DU-145 treated group, where the reduction in tumour size was noted to be 41% [43]. Additionally, it was demonstrated that the alkaloid showed anti-proliferative activities in human ovarian A2780 cancer cell in dose- and time-dependent manners [44]. In this circumstance, piperine induced the release of mitochondrial cytochrome *c* to cytosol, the activation of caspase-3 and -9, the PARP cleavage and the inactivation of p38/MAPK and JNK, so triggering the intrinsic pathway of apoptosis [44]. It is important to highlight the fact that piperine has selective cytotoxic autophagic/apoptotic efficacy on tumor cells whereas similar effects are not observed in normal cells at least at concentration < 250 µg/ml [8–10]. Additional beneficial effects have been reported induced by piperine, such as pain reduction, blood pressure lowering and increasing the absorption of nutrients [45–47].

Piperine indirectly interferes in cancer cell dissemination: effects on angiogenesis and extracellular matrix (ECM) degradation

Angiogenesis consists in the growth of new blood vessels from the pre-existing vasculature. In tumor, angiogenesis, which supplies oxygen and nutrients, is important for the proliferation as well as for the metastatic spread of cancer [48]. Piperine interferes with many aspects of angiogenesis in vitro and in vivo (Fig. 2c). For example, Doucette et al., by using cultures of human umbilical vein endothelial cells (HUVECs), showed that the alkaloid inhibited cell proliferation, migration, formation of tubule-like structures, and, it also stopped cellular cycle at G1/S phase [49]. As demonstrated by the same authors, the spice extract was also able to inhibit spouting process of rat aorta in ex vivo model and in vivo angiogenesis induced by breast cancer cell grafted on chick chorioallantoic membrane (CAM) [49]. At molecular level, in the context of anti-angiogenic properties, it seems that piperine selectively targets vascular endothelial growth factor/receptors (VEGF/VEGFRs) signal and phosphoinositide-3 kinase(PI3K)/Akt pathways [49–51]. On the basis of these experimental observations, piperine can be contemplated as an angiopreventive compound. This aspect could assume a certain pharmacological relevance; in fact, it is well known that targeting angiogenesis can be an important mechanism in cancer therapy as it reduces tumor proliferation by depriving the cancer of oxygen and

nutrients [52]. Interaction of cancer cells with their microenvironment plays a significant role in defining the severity of cancer [53]. Accordingly, evidences indicate that there is a crucial link among malignant cell dissemination, angiogenesis and ECM degradation. This last phenomenon is actuated by the proteolytic action of several enzymes including matrix metalloproteinases (MMPs), a family of Ca^{++}-and Zn^{++}-dependent endopeptidases [54, 55]. As emerged by experimental studies, piperine negatively modulates the expression and activation of MMPs corroborating the idea that the alkaloid, apart its direct anti-cancer effect, also indirectly hampers tumor spreading (Fig. 2c). As matter of fact, Hwang and co-workers by pre-treating human fibrosarcoma cell line (HT-1080) with phorbol-12-myristate-13-acetate (PMA), developed a high invasive phenotype of this cancer model due to enhanced expression of MMP-9 [56]. Then, the same authors demonstrated that piperine showed anti-invasive effects through inhibition of PKCα and ERK1/2 phosphorylation and reduction of NF-κB and AP-1 nuclear translocation. Consequently, all these molecular mechanisms down-regulated MMP-9 expression [56]. Many studies proposed that increasing in epidermal growth factor receptor 2 (HER2) dimerization could drive proliferation and migration of carcinomas by induction of MMP-2 and MMP-9 (also known as gelatinases A and B). For example, dimeric form of HER2 (with EGFR1 or HER3) has been identified as an important regulator of metastatic potential of human breast cancer [57]. Piperine significantly inhibited spreading of human breast cancer cells overexpressing HER2, by down-regulating gelatinases activation through NF-kB and AP-1 transcription factors inactivation and by interfering with ERK1/2, p38/MAPK/Akt axis [57]. Additionally, as demonstrated by Lai and co-workers, piperine suppresses lung metastasis of 4T1 murine breast cancer model by decreasing the expression of MMP-9 and MMP-13 [26].

Piperine disturbs redox homeostasis in cancer

ROS and free radicals represent a group of highly reactive molecules generated through a variety of sources including mitochondria, NADPH oxidases (Nox), xanthine oxidase (XO), and uncoupled endothelial nitric oxide synthase (eNOS), lipoxygenase, cyclooxygenase, and CYP-P450s enzymes [58–60]. They are involved in the regulation of a number of biological processes acting directly or indirectly as signalling factors [61, 62]. At physiological levels, ROS exist on stable equilibrium and can be implicated in cell survival or in defensive responses by means of enzymatic (catalase, proxidase and superoxide dismutase) and non-enzymatic molecules (i.e. glutathione, flavonoids and anti-oxidants such as vitamins A, C and E) [63, 64]. Notably, cells are able to preserve ROS homeostasis by balancing their production and elimination [65–67]. On the contrary, under metabolic stress, cellular amount of ROS increases with consequent oxidation of lipids, DNA and proteins [66–70], which in turn, can transform normal cells to malignant cells [64, 71, 72]. It is well known that cancer produces elevated levels of oxidative signals able to activate oncogenes and/or down-regulate tumor suppressors, which in turn, drive cell invasion and dissemination [71, 73–75]. Recent evidences indicate that phytochemicals and natural compounds including piperine, by means of their intrinsic anti-oxidative proprieties, can contribute to defensive responses against ROS [68, 70]. For example, low doses of piperine (not more than 200 µg/ml), by quenching ROS, attenuate oxidative stress and delay cancer development [68, 69]. As matter of fact, on mice model of lung cancer, oral administration of piperine decreased oxidative stress mediated by mitochondrial activities and by enhanced enzymatic and non-enzymatic defence systems [76, 77]. Moreover, in rat models of colon cancer induced by carcinogens such as 7,12-dimethyl benzanthracene, dimethyl aminomethyl azobenzene and 3-methyl cholanthrene, piperine was able to abrogate cellular ROS damages orchestrated by the above mentioned drugs [78]. All together, these observations seem to be in accordance with the concept that the modulation of intracellular ROS level can be proposed as a way to target oxidative stress responsible of tumourigenesis [79, 80]. In this context, high doses of piperine can also contribute in ROS generation causing cell death in many types of cancer cells [81]. Thus, depending on the context and on the piperine dosage, the alkaloid may act as anti-oxidant agent, abrogating or delaying tumorigenesis, or as pro-oxidant mediator, committing cancer cell to death [27, 81, 82]. For instance, in oral squamous carcinoma and in aggressive human rectal adenocarcinoma, piperine stimulated the generation of ROS, leading to dissipation of mitochondrial membrane potential, caspases activation and apoptosis induction [21, 81]. Similar conditions were also detected in hepatocellular carcinoma, in which it has been showed that piperine causes ROS-dependent apoptosis inhibiting the central molecule of the antioxidant defence system namely catalase, both in vitro in Hep G2 cells and in vivo, in dietilnitrosamine induced hepatocellular carcinoma at 5 mg/kg body weight of dosage [80]. In addition, in PC3 human prostate cancer cells, after treatment with piperine, it was observed an increase of intracellular levels of ROS and Ca^{++} followed by mitochondrial membrane depolarization and programmed cell death [83]. Of note, the hydrophobic nature and poor aqueous solubility can represent a limit for cell incorporation and bioavailability of the alkaloid. Thus, Jain and co-workers, in order to scavenge this limit, have recently demonstrated that piperine loaded in nanofibers results more permeable in HeLa and MCF7 cancer cells, causing cell death ROS-dependent [27]. Therefore, the

ability of piperine to induce oxidative stress and consequent apoptosis in cancer cells reinforce its potential chemopreventive ability (Fig. 2a, b).

Piperine inhibits cancer stem cells (CSCs)

CSCs represent a small subset of cancer cells with a high pro-metastatic phenotype; they show phenotypic and functional features comparable to normal stem cells, including self-renewal and differentiation ability [84]. As emerged by a number of recent papers, two "hallmarks of cancer" such as hypoxia and epithelial-mesenchymal transition (EMT) can contribute to the persistence of stem cells in cancer [85, 86]. Moreover, evidences indicate that CSCs are not only involved in cancer cell spreading but they are also responsible of chemotherapy and radiotherapy resistance [84, 87, 88]. Thus, it is plausible to think that the differentiation of these cells in a more mature/resting phenotype can assume a therapeutic significance. Some natural compounds are under investigation to test their ability in inducing stem cells differentiation [89–91]. For example, it has already demonstrated that piperine stimulates osteoblast differentiation acting on the expression of osteogenic marker genes by means of AMPK phosphorylation [91]. It is crucial to further explore the mechanism involved in the generation and perpetuation of CSCs in order to optimize efficient pharmaceutical approaches. Recent data indicate that several aberrant pathways are implicated in the maintaining of self-renewal and differentiation aptitude in CSCs including Wnt/beta-catenin, Hedgehog, Insulin-like growth factor, (IGF), Notch, PI3K/Akt/mTOR and NF-κB signaling [20, 92–94]. Interestingly, some of these signals are influenced by piperine and other natural compounds [95]. For example, piperine inhibits Wnt/beta-catenin signaling pathway in breast CSCs (Fig. 2c) especially when combined with curcumin [95, 96] and, as a consequence, it hinders the mammospheres formation, thus affecting their self-renewal ability [95, 97].

Piperine inhibits multidrug resistance and enhances the bioavailability of anti-cancer compounds

Development of multidrug resistance in cancer is a severe problem that restricts the use of chemotherapeutics in successful treatment [1]. Several factors participate, often in cooperation, in conferring multidrug resistance including EMT, CSCs, hypoxia, transforming growth factor-beta (TGF-β) and p-glycoprotein (P-gp) [1, 98, 99]. Currently, there are not experimental evidences regarding if and how piperine could act on repression of EMT, hypoxia, and TGF-β -multidrug resistance on cancer; by contrast, some investigations have shown that the alkaloid intervenes in P-gp and CSCs activities. P-gp is a 170 kDa membrane linked protein, member of the ATP-binding cassette (ABC) superfamily. The protein confers resistance by mediating the ATP-dependent efflux of a number of anticancer drugs [100, 101]. The use of many anti-cancer treatments such as vincristine, vinblastine, docetaxel, cyclophosphamide, flutamide, ifosfamide and paclitaxel causes overexpression of P-gp with a consequent induction of multidrug resistance [101]. It seems that piperine is able to suppress, or at least, down-regulate P-gp dependent drugs-resistance by competing with its ATP binding site. In support of this hypothesis, it has been recently demonstrated that two piperine analogues, namely Pip1 and Pip2, when co-administered with vincristine or colchicine or paclitaxel could reverse drug resistance in vitro in cervical and colon cancer cells overexpressing P-gp [102]. Metabolism, kinetics and bioavailability of xenobiotic agents (chemical drugs or natural compounds) depend, in part, on cytochromes P450 action (*CYP3A4, CYP1A, CYP1B1, CYP1B2, CYP2E1, CYP3A4,* etc.) [70, 103]. It has been recently demonstrated that bioavailability of anti-cancer drugs including docetaxel, etoposide, 5-fluorouracil, paclitaxel and rapamycin can be enhanced by piperine both in vitro and in vivo (Fig. 2d) [16, 57, 104–106]. For example, in 5L rat hepatoma, piperine mediates deficiency of benzo(a)pyrene (BP) metabolism through a direct interaction with CYP1A1 enzyme and, consequently, it abolishes cancer aggressiveness [107]. Apart from influencing CYP-P450 isoforms, piperine modulates many other drugs metabolizing enzymes (DMEs) which include uridine diphosphate-glucoronyl transferase (UDP-GT), UDP-glucose dehydrogenase (UDP-GDH), 5-lipooxygenase, cyclooxygenase [108]. About that, oral administration of piperine in mice model of lung carcinogenesis not only causes a significant activity reduction of phase-I enzymes (i.e. CYP-450 family), but also, causes a concrete increase in glutathione-metabolizing enzymes including glutathione peroxidase (GPX) and glutathione reductase (GR) [109]. As mentioned above, piperine is also capable to enhance the bioactivity of some natural compounds, such as curcumin, resveratrol and thymoquinone (TQ). Many studies have reported that the phytochemicals, above mentioned, also possess chemopreventive actions [110–131] (Table 2). Regarding curcumin, it has been shown that this spice, in combination with piperine, has greater antimetastatic effect in vitro on breast cancer lines MCF-7 and MDA-MB-231 [95] as well as in diethylnitrosamine-induced hepatocarcinoma cells [132]. Concerning the increased efficacy of resveratrol, piperine substantially improved the bioavailability of this aromatic compound, mostly through an inhibitory effect on UDP-glucuronosyltransferase [133]. In fact, for its elimination from the body, as other phenols, resveratrol is biotransformed through the glucuronidation process [134]. TQ is a natural aromatic compound, predominated present in *Nigela sativa* volatile oil; it exhibits a wide spectrum of positive features including anti-angiogenic and anti-cancer

Table 2 Summary of the main chemopreventive actions of some natural compounds

Compounds	Main effects	References
Piperine	Interruption of cell cycle at different checkpoints by downregulation of cyclins through the inhibition of CDKs activity; promotion of cancer cell death through activation of apoptosis and autophagy; prevention of angiogenesis by targeting VEGF/VEGFRs signal pathway; unbalance of ROS homeostasis with consequent cell death; quenching multi-drug resistance	[22, 39, 41, 43, 44, 49, 50, 68, 69, 78, 102]
Curcumin	Arrest of the cell cycle at the G1/S transition by upregulating the expression of the Cip/Kip protein family of CDK inhibitors; induction of autophagic and apoptotic cell death; suppression of angiogenesis by decreasing the levels of bFGF, VEGF, angiopoetin-1 and 2, COX-2, MMP-9 and AP-1; inhibition of tumor growth and metastasis by maintaining of NF-kB in its inactive form	[110]
Resveratrol	Arrest of cell cycle at different checkpoints by modulating the CDK inhibitor; increase of ROS generation provoking apoptosis; inhibition of EMT by suppressing both the PI3 K/AKT/NF-κB pathway and the EMT-related gene expression; suppression of the initiation, and progression of carcinogenesis by decreasing the expression of ERK and VEGF and by reducing the phosphorylation of Her-2 and EGF-R	[115]
Thymoquinone	Interruption of cell cycle at different checkpoints due to decreased cyclins levels; induction of apoptosis and autophagy; blocking of a tumor angiogenesis and tumor growth via inhibited activity of NF-kB and its downstream targets including VEGF; reduction of invasion and metastasis by downregulating the transcriptional activity and expression of TWIST1 promoter; inhibition of cell proliferation by downregulating STAT3 downstream targets	[124]

VEGF/VEGFRs vascular endothelial growth factor/vascular endothelial growth factor receptors, *PI3 K)/Akt* phosphoinositide 3-kinase/protein kinase B, *CSC* cancer stem cell, *COX-2* cyclooxygenase-2, *FGF-2* fibroblast growth factor-2, *EMT* endothelial-mesenchymal transition, *Her-2* human epidermal growth factor receptor 2, *EGF-R* epidermal growth factor receptor, *TWIST1* twist family BHLH transcription factor 1, *STAT3* signal transducer and activator of transcription 3

effects, inhibiting tumour growth and angiogenesis both in vitro *and* in vivo [135] without toxicity on healthy cells [136]. It has been demonstrated that a combination of TQ and piperine can work synergistically in inhibition of breast cancer growth in vitro and in vivo (syngraft model). This combination seems to act mainly by apoptosis induction, angiogenesis inhibition and by shifting the immune response toward the modulation of the immune system [137].

Piperine metabolism and availability

Piperine was extracted as a yellow crystalline compound with a melting point of 128–130 °C and its chemical structure is identified as piperoylpiperidine with the chemical formula of $C_{17}H_{19}NO_3$ [138]. Piperine is a very weak base, which upon acid or alkali hydrolysis decomposes to a volatile basic piperine, known as piperidine ($C_5H_{11}N$), and piperic acid ($C_{12}H_{10}O_4$) [139].

Piperine metabolism was elucidated in studies performed in animal models [140, 141]. When orally administered, piperine is rapidly and almost completely absorbed through the gastrointestinal trait and could be detected in plasma after 15 min [142]. The maximum plasma concentration was reached at 6 h and its half-life was to be 18 h [143]. Only 3% of not absorbed piperine was directly excreted in the feces. Piperine did not undergo any metabolic changes during absorption and it is rapidly metabolized by liver while its metabolites are excreted by urine. It has been demonstrated that piperine is able to interact with several enzymes involved in drugs metabolism and it is able to affect metabolic pathways and processes such as oxidative phosphorylation. The final result is a slowdown of drugs metabolism and biodegradation with a consequent more availability for pharmacological actions [141].

Conclusion and perspective

Current available drugs for treating cancer are often expensive, toxic, and little effective. Piperine possesses high potential chemopreventive properties due to induction of cell cycle arrest, increased autophagy and apoptosis, as well as redox homeostasis unbalance. Furthermore, it inhibits angiogenesis and ECM degradation. Therefore, the alkaloid directly and indirectly affects tumor cell survival and quenches invasion and metastasis processes, without significant toxic effects on normal cells [8–10]. Additionally, piperine enhances the therapeutic efficacy of the current available anti-cancer drugs and represents a good adjuvant to certain phytochemical compounds including curcumin, resveratrol, thymoquinone. In summary, piperine has a number of health effects and therapeutic properties; nevertheless, its biological applications are limited due its hydrophobic nature. In fact, piperine is slightly soluble in water (40 mg/L at 18 °C) [144]. This facet is the rate-controlling step in the absorption process and limits its clinical employment. However, in order to improve the bioavailability of this compound, several attempts have been made to develop new formulations. Among the various approaches, nanoparticles, liposomes,

microspheres and self-emulsifying drug delivery systems have been developed and demonstrated to ameliorate its bioavailability, also reducing its toxicity [138, 145–148].

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest. All authors have read and approved the manuscript.

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Review

Mechanisms of Chemopreventive and Therapeutic Proprieties of Ginger Extracts in Cancer

Mariia Zadorozhna and Domenica Mangieri *

Department of Medical and Surgical Sciences, University of Foggia, Via Pinto 1, 71122 Foggia, Italy; mariia.zadorozhna@unifg.it
* Correspondence: domenica.mangieri@unifg.it

Abstract: Ginger (Zingiber officinale Roscoe, family: Zingiberaceae), originating in South-East Asia, is one of the most used spices and condiments for foods and beverages. It is also used in traditional medicine for many human disorders including fever, gastrointestinal complications, arthritis, rheumatism, hypertension, and various infectious diseases due to its anti-inflammatory, antioxidant, antimicrobial, and antiemetic properties. Intriguingly, many recent studies evidenced the potent chemopreventive characteristics of ginger extracts against different types of cancer. The aim of this work is to review the literature related to the use of ginger extracts as a chemotherapeutic agent and to structure the cellular and molecular mechanisms through which ginger acts in different cancer types. Data summarized from experiments (in vitro or in vivo) and clinical studies, evidenced in this review, show that ginger derivatives perpetrate its anti-tumor action through important mediators, involved in crucial cell processes, such as cell cycle arrest, induction of cancer cell death, misbalance of redox homeostasis, inhibition of cell proliferation, angiogenesis, migration, and dissemination of cancer cells.

Keywords: ginger extracts; chemoprevention; chemotherapy; natural compounds

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1. Introduction

Cancer, being a multifactorial disease, is the second biggest cause of death in the world despite a great development of different types of its treatment [1]. Therapeutic options against cancer include surgical procedure, radiation therapy, chemotherapy as well as target and gene therapy [2]. Since the currently available treatment options are often accompanied by severe toxicity and side effects, consequently, researchers are consistently searching for new therapeutic solutions [3].

In this regard, 50% of approved cancer therapeutic agents are derived from natural products and, secondarily, medicinal plants metabolites have demonstrated a great perspective as a source of anticancer and chemopreventive compounds [4]. Composts isolated from edible plants have the advantage of low toxicity profiles and can simultaneously target multiple signaling pathways [5]. Therefore, dietary natural products can provide novel and fascinating preventive/therapeutic options for different kinds of neoplasia.

Ginger is known for having more than 60 active compounds, broadly divided into volatile and nonvolatile compounds [6]. Volatile components include hydrocarbons, meanwhile rhizome from ginger contains nonvolatile pungent phenolic compounds like 6-gingerol, 6-shagol, 6-paradol, and zingerone [7] (Figure 1, Table 1).

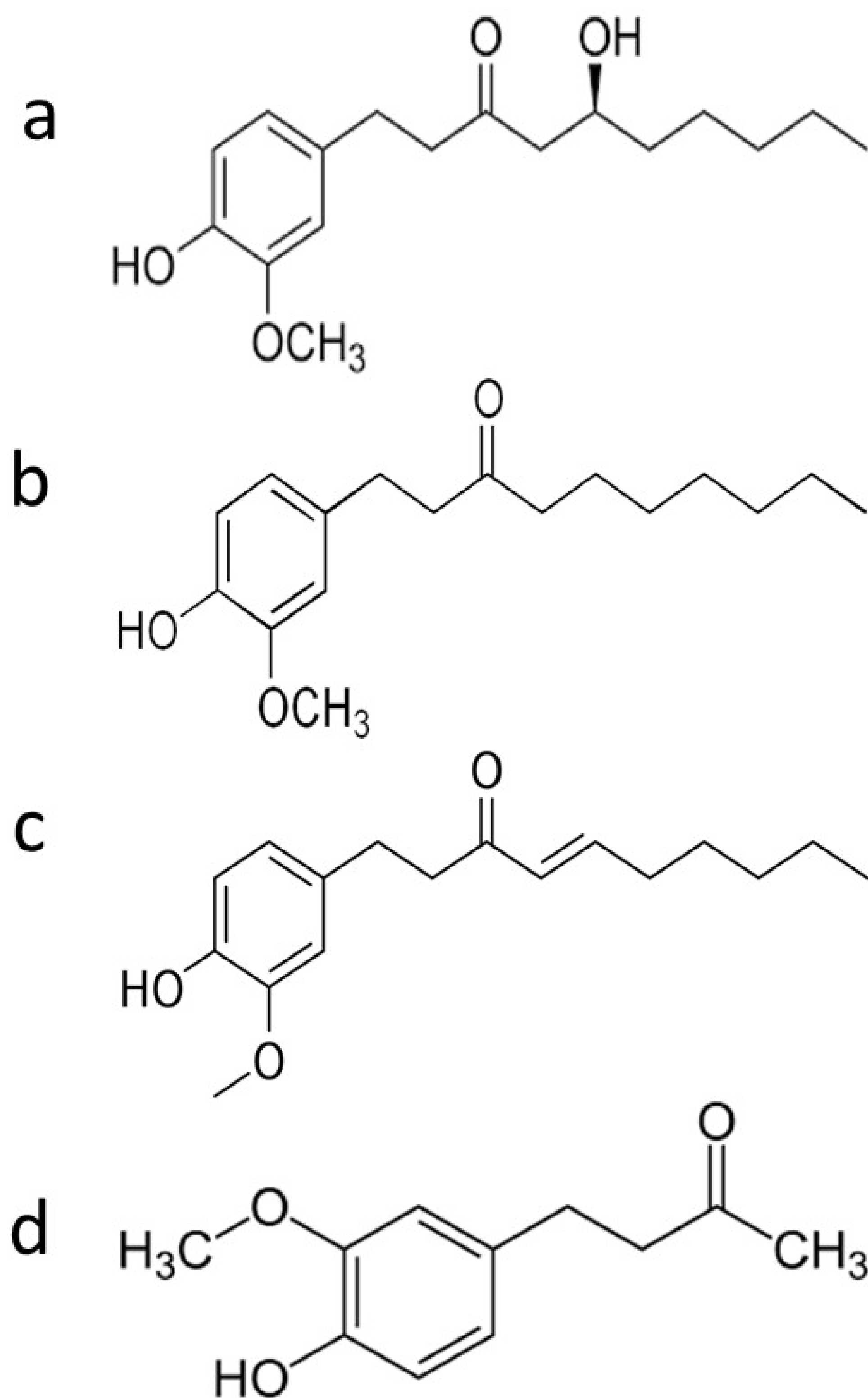

Figure 1. Chemical structure of main phenolic compounds of ginger: (**a**) 6-gingerol (1-[4′-hydroxy-3′-methoxyphenyl]-5-hydroxy-3-decanone), (**b**) 6-paradol, (**c**) 6-shogaol, and (**d**) zingerone.

Table 1. Chemopreventive activities of phenolic compounds of ginger.

Phenolic Ginger Compounds	Chemopreventive Activities	References
6-gingerol	Blockage of the cell cycle at G2/M phase; decrease of cells in the SubG0 phase; depolarization and potential subsequent deterioration of the mitochondrial membrane; induction of apoptosis; inhibition of angiogenesis; induction of growth suppression; enhancement the doxorubicin efficacy	[8–12]
6-paradol	Reduce blood glucose	[7]
6-shogaol	Arrest of the cell cycle in G2/M phase; decrease levels of STAT3 and NF-κB-regulated target genes including cyclin D1; induce apoptosis; downregulation of surviving; decrease tumor volume and tumor burden; restore wild type p53 function; provoke autophagy; inhibit phase I enzymes (Cyt-p450 and Cyt-b5); increase phase II enzymes (GST, GR, and GSH); reduce the cleavage of Notch1	[13–17]
Zingerone	Inhibition of TGF-β1 induced epithelial-mesenchymal transition, migration, and invasion	[18]

These exact compounds have been studied for their anti-bacterial, antioxidant, and anti-inflammatory properties [19]. Ginger phenolic compounds especially have also shown anti-tumor properties [9,20,21] (Figure 2). In this review, we will more deeply discuss the chemopreventive molecular mechanisms of ginger derivatives including arrest of the cell cycle, induction of cancer cell death, misbalancing of redox homeostasis, inhibition of cell proliferation, angiogenesis, migration, and dissemination of cancer cells in different cancer types (Table 2, Figure 3).

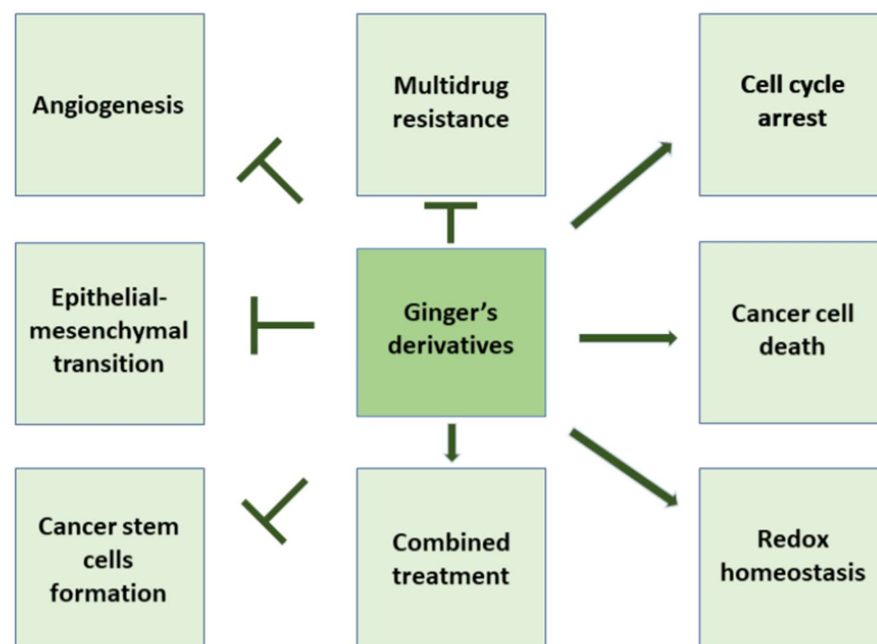

Figure 2. Schematic anti-cancer action of ginger and its phenolic derivatives. This natural compound reduces cancer cell proliferation, arrests cell cycle, causes an imbalance in cellular redox homeostasis, and induces cell death. Additionally, ginger derivatives inhibit angiogenesis, EMT, and CSCs. Furthermore, they decrease multidrug resistance and enhance chemopreventive effects.

Table 2. Effects of ginger in different types of cancer.

Tumor Entity	Functions of Ginger	References
Breast cancer	Blockage of the cell cycle at G2/M phase; Induction of typical apoptotic changes in nuclear morphology, chromatin condensation and fragmentation, membrane shrinkage and blebbing; enabled autophagy followed by caspase-independent apoptosis; induction of autophagy	[16,18,22]
Prostate cancer	Arrest of cell cycle in the G1 phase with subsequent decrease in S and G2/M through p21 dependent pathway; downregulation of MRP1 and GST-protein expression	[23,24]
Ovarian cancer	Suppressed production of NF-κB regulated angiogenic factors; p53 stimulation of apoptosis through Bcl-2 elimination	[25,26]
Colon cancer	Arrest of cell cycle at different check points by inhibition of cyclin dependent kinases and activation of cell cycle check points; upregulation of p21 expression; reverse of EMT to Mesenchymal–epithelial transition (MET) through the upregulation of miR-200c	[17,19,27,28]
Hepatocellular carcinoma	Arrest of cell cycle at the G2/M phase; inhibition of the PI3K/AKT/mTOR and STAT3 signaling pathways; inhibition of Bcl-2 expression and up-regulation of Bax, cytochrome c, caspase-9 and -3 protein expressions	[21,29]
Gastric adenocarcinoma	Interruption of cell cycle at different check points; mediation of mitochondrial pathway of apoptosis; unbalance ROS homeostasis and induction of apoptosis	[30]
Non-small lung epithelium cancer	The loss of mitochondrial membrane potential of that leads to increase in Bax/Bcl-2 ratio and activation of mitochondrial death cascade	[31,32]
Melanoma	Induction of caspase independent cell death via the inhibition of ERK1/2, p38 and Akt signaling pathway	[33]
Endometrial adenocarcinoma	Induction of apoptosis by increasing the expression of p53 and Bax and simultaneously decreasing the expression of Bcl-2	[34]
Cervical cancer	Induction of typical apoptotic changes in nuclear morphology, chromatin condensation and fragmentation, membrane shrinkage and blebbing	[35]
Lung cancer	Sensitization of TRAIL-induced apoptosis by inhibiting autophagy flux	[36]
Head and neck squamous carcinoma	Increase in apoptotic death by downregulation of surviving; inhibition of mutant p53 Bcl-2 expression, and increased expression of Bax, regulation of Bax/Bcl-2 ratio which induce cell apoptosis	[37,38]
Pancreatic cancer	Activation of AMPK, a positive regulator of autophagy, and inhibition mTOR, a negative autophagic regulator; unbalance ROS homeostasis and induction of autosis	[39]

Molecular action of ginger and its derivatives in different kinds of cancer.

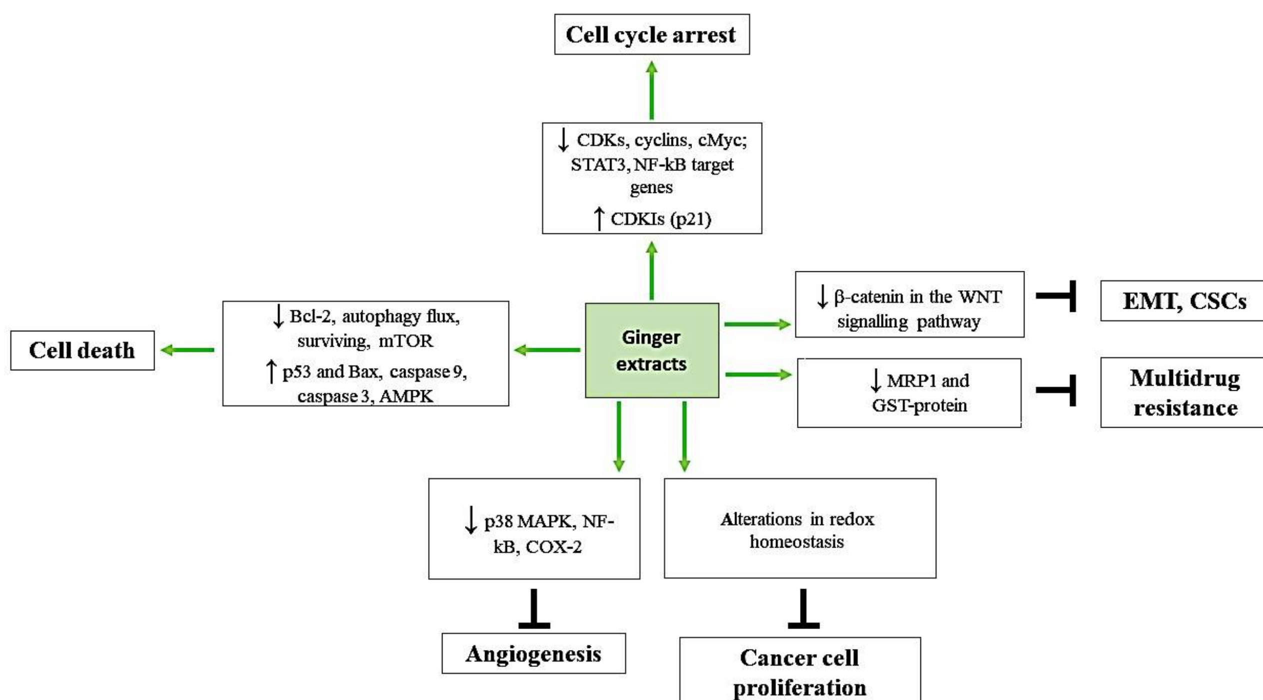

Figure 3. A schematic diagram showing main molecular targets of ginger derivatives. The natural compound participates in the cell cycle arrest by inhibiting the expression of cyclins, CDKs, and levels of STAT3, NF-kB target genes, and by activation of cell cycle check points and increased expression of p21. Moreover, by increasing Bax/Bcl-2 ratio outburst of cytochrome C, activating AMPK, and by decreasing autophagy flux and survivin expression, ginger derivatives provoke cancer cell death. They participate in the alteration of redox homeostasis, which stops cancer cell proliferation. By blocking activation of p38 MAP kinase (p38 MAPK) and NF-kB, ginger derivatives inhibit COX-2 expression and as a result block angiogenesis. Ginger extracts decrease β-catenin in the WNT signaling pathway, which leads to the inhibition of gene transcription, involved in EMT and CSCs, and, additionally, downregulate MRP1 and GST-protein expression, involved in multidrug resistance.

2. Ginger Derivatives and Cell Cycle Arrest

Cell cycle is critical to maintaining cell proliferation and tissue integrity; therefore, it is thoroughly controlled in defined checkpoints by specific proteins and kinases that include cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs) [30]. Deregulation of cell cycle and/or its arrest are often responsible for cancer onset and progression [30]. Experimental studies demonstrated that some ginger derivates were able to modulate cell cycle progression as a part of their chemopreventive mechanism [40] (Figure 2) For example, in recent work regarding the treatment of breast cancer cells (MCF-7 and MDA-MB-231) with 6-shogaol, the arrest of the cell cycle in G2/M phase was reported in both monolayer and cancer-stem cell-like spheroids, and in the last experimental setting, 6-shogaol also interfered with the stem cell self-renewal pathway [13]. In another study, ginger extract arrested cell cycle at G0/G1 and G2/M phases in the HT 29 colon cancer cell line at a low concentration (455 μg/mL), whereas in the HCT116 colon cancer cell line, the same effect was reached at a higher concentration (496 μg/mL) due to the inhibitory effect on CDKs [41]. Furthermore, in the HT29 and HCT116 colon cancer cell lines, ginger extract treatment caused a significant reduction in cyclin D1 gene expression coupled with down-regulation of mTOR and Wnt/β-catenin pathways and consequent cell cycle arrest in the G0/G1 phase [41,42]. In addition, it was reported that human colon cancer cells (HCT116) treated with R. stricta (CAERS) and crude flavonoid extracts from Z. officinale (CFEZO) acted synergistically in cycle progression by inhibiting cMyc and the Cdk4/cyclin complex while upregulating p21 expression, a transcriptional target of p53 [43]. Saha and co-workers observed that cancer prostate cell lines (human PC3; DU145; LNCaP

and murine HMVP2) treated with 6-shogaol, showed decreased levels of several signal transducer and activator of transcription 3 (STAT3) and NF-κB-regulated target genes including cyclin D1 [14]. In addition, zerumbone (a sesquiterpene derived from the ginger plant Zingiber zerumbet) caused the arrest of cell cycle in the G1 phase in human prostate cancer cell line (DU-145) culture [14]. Furthermore, zerumbone caused a cell cycle arrest at G2/M (in a dose-dependent manner) by inhibiting PI3K/AKT/mTOR and STAT3 in hepatocellular carcinoma (human HepG2, Hep3B, Sk-Hep-1, SNU-182, SNU-449, Huh-7, and MHCC-LM3 cell lines, and murine Hepa1 cell line) [25]. The same compound impeded shunting of glucose-6-phosphate through the pentose phosphate pathway, thereby forcing tumor cells to undergo a cell cycle arrest. In human gastric adenocarcinoma (AGS), the 6-gingerol treatment showed a notable increase in the percentage of cells in the G2/M phase, accompanied by a resultant decrease of cells in the SubG0 phase [8].

Another study showed that zerumbone (ZER) administration significantly retarded the growth of orthotopic MDA-MB-231 xenografts in severe combined immune-deficient (SCID) mice [44]. The antitumor effect of ZER in vivo was accompanied by reduced cell proliferation as evidenced by Ki-67 (proliferation marker) expression and increased apoptosis. Additionally, ZER administration was well-tolerated by the mice and did not cause weight loss or any other side effects.

Based on the above-mentioned observations, it seems evident that ginger derivatives interfere with the proliferation and cell cycle of cancer cells by arresting cell cycle in G0/G1 or G2/M phases, by significantly reducing the cyclin D1 gene expression, by upregulating p21 expression, and by inhibiting PI3K/AKT/mTOR and STAT3.

3. Ginger and Cellular Death

Apoptosis, or programmed cell death, evolved as a rapid and irreversible process to efficiently eliminate dysfunctional cells [31]. Apoptosis is usually executed in two ways: Mitochondria-mediated intrinsic pathway and death receptor-mediated extrinsic route. In this process are involved cysteine-aspartate proteases (caspases) and the Bcl-2 family proteins (e.g., Bax, Bcl-2) [29,32]. Moreover, it is well known that in pathological conditions such as cancer, alterations/mutations in the p53 gene are one of the main causes of apoptosis changes [33]. As matter of fact, studies effected by Pashaei-Asl et al. showed that the treatment of ovarian cancer cell line SKOV-3 with ginger extract for 48 h caused the decrease of Bcl-2 gene expression, and the subsequent p53-induced apoptosis [45]. In another study, depolarization of the mitochondrial membrane and its potential subsequent deterioration ($\Delta\Psi m$) were observed after 6-gingerol administration within the human gastric adenocarcinoma cell line (AGS) [8]. Interestingly, disruption of the mitochondrial permeability through the transition pore with decrease in $\Delta\Psi m$ is one of the pivotal events in cell response to apoptotic stimuli [34]. Mitochondria-mediated apoptosis in 6-gingerol-treated AGS cells was followed by cytochrome c release, elevation in the Bax/Bcl-2 ratio, and activation of caspases-3 and -9 [8]. Aqueous extract of ginger (GAE) induced cellular apoptosis and disrupted cellular interphase microtubules also within human non-small lung epithelium cancer (NSCLC) A549 cell lines, by increasing the Bax/Bcl-2 ratio and activating the mitochondrial death cascade [46]. In addition, 6-shogaol induced apoptosis in hepatocarcinoma cell lines (Hep-2) by loss of cell viability, enhanced ROS production, lipid peroxidation resulted in altered mitochondrial membrane potential, and increased DNA damage [15]. In particular, the prooxidant role of 6-shogaol seemed to inhibit Bcl-2 expression accompanied by an up-regulation of Bax, cytochrome c, released by altered mitochondria, and caspases-3 and -9 activation [15]. Similarly, β-Elemene, another extract from the ginger plant, triggered apoptosis in NSCLC through the cytochrome-c mitochondrial release, mediating the intrinsic apoptotic pathway [47]. Furthermore, torch ginger (*Etlingera elatior*, EE) induced caspase-independent cell death in mouse B16 melanoma cells through the inhibition of the ERK1/2, p38, and Akt signaling pathway [37]. In fact, it is well-known that the PI3K/Akt, ERK1/2, and p38 MAPK signaling pathways are crucial in the context of DNA-damaging drug-induced apoptosis [38]. Terpenoids present

in the Steam Distilled Extract of Ginger (SDGE) induced apoptosis in endometrial cancer cells (ECC-1 and Ishikawa cell lines) at IC50 of 1.25 µg/mL by increasing the expression of p53 and Bax and simultaneously decreasing the expression of Bcl-2 by 90% [48].

Survivin is a member of the inhibitor of apoptosis (IAP) family and results in being up-regulated in different human cancers [35]. Interestingly, over-expression of this protein is associated with inhibition of apoptosis, resistance to chemotherapy, and a higher aggressiveness of tumors [49]. In this context, recent study showed that 6-shogaol, at 20 µM and 40 µM, provoked downregulation of survivin in head and neck squamous cell carcinoma (HNSCC) cell lines and consequently a significant increase in apoptotic death [16].

A very important fact is that there are some results of in vivo experiments confirming the positive role of ginger derivatives in apoptosis induction within cancer. In this setting, oral squamous cell carcinoma induced by painting with 0.5% 7,12-dimethylbenz[a]anthracene-induced (DMBA-induced) in hamster buccal pouch (HBP) (male golden Syrian hamsters) evidenced over-expression of the mutant form of p53 and Bcl-2 coupled with decreased expression of wild type p53 and Bax [17,50]. Oral treatment with 6-shogaol (at 20 mg/kg of body weight) showed significantly decreased tumor volume and tumor burden, restored wild-type p53 function, and activation of apoptotic stimuli [17].

Methanolic extract of *Zingiber officinale* rhizome (ZOME) induced morphological changes such as cell shrinkage and nuclear condensation demonstrating apoptotic properties of ZOME within cervical cancer HeLa and breast cancer MDA-MB-231 cell lines 1. Moreover, apoptosis of these cancer cell lines was gradually raised with an increasing order of concentration of extract, which revealed dose-dependent apoptosis [51].

Autophagy is a self-destructive process important for balancing sources of energy in the embryo development and as a response to several triggers of cellular stress (e.g., deprivation of growth factors/nutrients, inhibition of proteasome, inhibition of receptor tyrosine kinases/Akt/mammalian target of rapamycin (mTOR) signaling, and unbalance of ROS homeostasis) and is characterized by a cascade of events including degradation of cytoplasmic proteins or entire organelles [36,52,53]. Interestingly, the correlation between autophagy and cancer is controversial; in this context, it seems that autophagy could act as a tumor suppressor, provoking a programmed cell death of type II [22] and/or as tumor activator, directly affecting the cell–matrix focal adhesions (FAs), essential for efficient migration and invasion of cells [39].

Interestingly, co-treatment with gingerol and TRAIL of TRAIL-resistant A549 adenocarcinoma cells increased the LC3-II and p62 levels, that attested the inhibition of autophagy [54]. The gingerol treatment strongly enhanced apoptosis in TRAIL-resistant A549 cells, which was confirmed by the intracellular apoptosis indicator cleaved caspase-3. The results of this study suggested that gingerol sensitized TRAIL-induced apoptosis in A549 lung adenocarcinoma cells by inhibiting autophagy flux.

The semi-synthetic analogue SSi6, generated after chemical modification of the 6-gingerol molecule, using the acetone-2,4- dinitrophenylhydrazone (2,4-DNPH) reagent, enhanced selective cytotoxic effects on MDA-MB-231 (Triple negative breast cancer, (TNBC)) cells [55]. Remarkably, unlike the original 6-gingerol molecule, SSi6 enabled autophagy followed by caspase-independent apoptosis in tumor cells. A time-dependent association between SSi6-induced oxidative stress, autophagy, and apoptosis was reported. Initial SSi6-induced ROS accumulation (1 h) led to autophagy activation (2–6 h), which was followed by caspase-independent apoptosis (14 h) in TNBC cells. Additionally, the data showed that SSi6 induction of ROS accumulation played a key role in the promotion of autophagy and apoptosis [55]. Another experiment showed that breast cancer cells MCF-7 and MDA-MB-231 after 6-shogaol treatment underwent cell death, exploiting the autophagy route proved by cytoplasmic vacuole formation as well as the recruitment and cleavage of the microtubule-associated protein LC3 [13].

Another recent study showed that 6-Gingerol treatment in the human lung cancer cell line (A549) and in A549 tumor xenografts could increase the number of autophagosomes, ROS, and iron concentration, decrease the survival and proliferation rate of A549 cells, and

significantly decrease tumor volume and weight [56]. Interestingly, 6-Gingerol treatment significantly suppressed USP14 expression, indicating that 6-Gingerol promoted autophagy effected by inhibition of USP14-Beclin 1. Remarkably, daily oral feeding of 100 mg/kg body weight of ginger extract (GE) inhibited growth and progression of PC-3 (prostate cancer) xenografts by approximately 56% in nude mice, as shown by measurements of tumor volume [57]. Tumor tissue from GE-treated mice showed reduced proliferation index and widespread apoptosis, as determined by immunoblotting and immunohistochemical methods, compared with controls.

Also, recent research demonstrated that the human pancreatic cancer (Panc-1) cell line treated with the extract of *Syussai ginger* (SSHE) revealed several features that were not observed in classical-type autophagy, including nuclear shrinkage, focal membrane rupture, electron dense mitochondria, empty vacuoles, and focal perinuclear swelling [58]. It appeared that these morphological features coincided well with the recently discovered form of cell death, "autosis", which is a Na^+ and K^+ -ATPase-regulated form of cell death [23]. SSHE markedly increased the LC3-II/LC3-I ratio, decreased SQSTM1/p62 protein, and enhanced vacuolization of the cytoplasm in Panc-1 cells. So, SSHE inhibited cell proliferation and subsequently induced the autotic death of pancreatic cancer Panc-1 cells.

To sum up, the chemopreventive effect of ginger derivatives may be expressed by its ability to enhance some types of cellular death in cancer, like apoptosis, autophagy, and autosis by elevating Bax/Bcl-2 ratio, releasing cytochrome c, activating caspases-3 and -9, and downregulating the survivin.

4. Ginger, Its Constituents, and ROS Balance

Reactive oxygen species (ROS) are a group of highly reactive molecules generated through a variety of sources (mitochondria, NADPH oxidases (Nox), xanthine oxidase (XO), and uncoupled endothelial nitric oxide synthase (eNOS), lipoxygenase, cyclooxygenase, and CYP-P450s enzymes) [59–61]. Elevated ROS rates have been detected in almost all cancers, where they promote many aspects of tumor development and progression. However, tumor cells also express increased levels of antioxidant proteins to detox from ROS, suggesting that a delicate balance of intracellular ROS levels is required for cancer cell function [62].

A challenge for novel therapeutic strategies will be to direct the ROS signaling towards ROS-induced apoptotic route. In this scenario, recent studies showed that ZOME scavenged the ROS actions, in a dose-dependent manner, both in human cervical cancer (HeLa) cells and in breast cancer (MDA-MB-231) cells [51]. Another study reported that after treatment of AGS cells with 6-gingerol, an increase in the level of reactive ROS led to a decrease in mitochondrial membrane potential and consequent induction of apoptosis [8]. Also, the incubation of DU-145 prostate carcinoma cells with zerumbone led to a reduction of cell viability, in a dose- and time-dependent manner, by increasing the ROS production [63]. Additionally, Akimoto and co-workers demonstrated that the extract of Syussai ginger (SSHE) had a strong inhibitory effect on cell growth as well as pro-apoptotic activity in pancreatic cancer in vitro [58]. In particular, the authors showed that ROS production was suppressed in SSHE-treated Panc-1 cells at early stages that might be due to the antioxidant properties of the ginger extract [64]. However, prolonged treatment of cells with SSHE caused a marked increase in ROS production, which induced autotic cell death. The extract was also effective under hypoxic conditions, which inevitably develop in all solid tumors to varying degrees and influence the resistance of tumor cells to radiotherapy and conventional chemotherapy [64]. Recently, Kathiresanet et al. discovered that 6-shogaol (20 mg/kg body weight) had a potent anticancer activity against DMBA-induced oral carcinogenesis in the HBP model by restoring antioxidant levels, thereby preventing lipid peroxidation. In addition, 6-shogaol was also able to inhibit phase I enzymes (Cyt-p450 and Cyt-b5) and increase phase II enzymes (GST, GR, and GSH) that enhanced the detoxification, thereby preventing the carcinogenesis [17].

Another interesting clinical study had the principal objective to examine the antioxidant activity of ginger extract orally administered as a daily supplement in newly diagnosed solid tumor patients receiving moderate-to-high emetogenic potential chemotherapy [65]. All participants were women, of whom 39 patients (91%) were diagnosed with breast cancer who received anthracycline-based regimen, 24 patients (56%) were diagnosed with stage II, and 13 patients (30%) diagnosed with stage III. In all, 90% of patients had a good performance status (ECOG = 0). A daily supplement of ginger extract, started 3 days prior to chemotherapy, showed significantly elevated antioxidant activity and reduced oxidative marker levels in patients who receive moderate-to-high emetogenic potential chemotherapy compared to a placebo [65]. In subsequent cycles of chemotherapy, patients seemed to have significantly elevated oxidative defense status based on their higher blood levels of Cu-Zn superoxide dismutase (CuZn-SOD), catalase (CAT), glutathione (GSH/GSSG), and GPx couplet with significantly reduced levels of malondialdehyde (MDA) and NO_2-/NO_3- after continuously receiving ginger extract. This effect was not observed in patients who received placebos. Furthermore, patients taking ginger extract continuously were inclined to increase antioxidant enzyme blood levels and decrease oxidative stress blood level [65].

In summary, ginger derivatives could play an important role in maintaining redox homeostasis: In some cases, by decreasing the quantity of ROS-induced tumor-promoting events, and in other cases, in contrast, by increasing oxidative stress and provoking cell death.

5. Ginger and Angiogenesis

Angiogenesis, the formation of new blood vessels from pre-existing endothelium, depends on complex cellular activities, such as extracellular matrix degradation, proliferation and migration of endothelial cells, and morphological differentiation of endothelial cells to form tubes. Thus, this phenomenon is tightly controlled by positive factors such as vascular endothelial growth factor (VEGF) and negative regulators including endostatin, thrombospondin, etc., [66,67]. Neovascularization is fundamental in a variety of physiological processes such as embryonic development and pregnancy [68]. On the other hand, angiogenesis is a crucial event for tumor progression and metastatic cascade, therefore many cancer therapies are directed against the tumor-associated vasculature [69–71]. Recent observation showed that a series of natural compounds, including ginger extracts, were proposed as antiangiogenic/angiopreventive substances both in in vitro and in vivo [20,26]. In this contest, it was demonstrated that 6-gingerol was able to inhibit the proliferation and tube formation of human umbilical vein endothelial cells (HUVECs) in response to VEGF or bFGF [9]. Also, 6-gingerol strongly inhibited sprouting of endothelial cells in the rat aorta model and angiogenesis in the mouse cornea in response to VEGF; while in the mouse model of melanoma, i.p. administration of the above-mentioned ginger extract reduced the number of lung metastasis, with the preservation of apparently healthy behavior [9]. Kim and their collaborators showed that in phorbol ester-stimulated mouse skin, 6-gingerol was capable of inhibiting tumor promoter-induced activation of AP-1 and COX-2 expression by blocking the activation of p38 MAP kinase (p38 MAPK) and NF-κB [72]. Since p38 MAPK, NF-κB, and COX-2 are involved in angiogenesis, the anti-angiogenic activity of 6-gingerol might be due to blocking their activation.

Moreover, the use of CAM assay showed that ginger extracts were capable to reduce neovascularization as well as blood vessel diameter in a dose-dependent manner [73]. The importance of 6-gingerol in angioprevention and cancer treatment was also supported by further experimental evidence that demonstrated that the ginger extract was a potent inhibitor of endothelial cell proliferation as tube-like formation in vitro and in vivo, directly inhibiting the growth of rat YYT colon cancer cells or mouse MS1 endothelial cells in response to the growth factors derived from another colon cancer cell line (mouse CT26) [74]. Interestingly, there is an inverse dose-dependent relationship between proliferation and concentration of the ginger extract used [74].

NF-κB, as well as IL-8, plays an important role in tumorigenesis, given its ability to control the expression and function of numerous genes involved in cell proliferation, sustained angiogenesis, and evasion from apoptosis. Different tumor types, including ovarian cancer, have been shown to express high constitutive NF-κB activity [75]. It was shown that 6-gingerol treatment of cultured ovarian cancer cells induced serious growth suppression by inhibiting NF-kB activation and decreasing the VEGF and IL-8 secretion [10].

In summary, ginger derivatives seem to be potent anti-angiogenic substances that point to a possible role in preventing cancer from becoming malignant, presumably by selective inhibition of neovessel formation in tumor sites.

6. Cancer Stem Cells, Epithelial-Mesenchymal Transition and Ginger

In many tumors, a subpopulation of cells named cancer stem cells (CSCs) is involved in dissemination through their stemness properties. In fact, CSCs play a critical role in metastatic potential, resistance to chemotherapies, as well as the relapse of malignancies [76–79]. These cells are frequently identified in various tumors, including brain, pancreas, liver, ovary, colon, lung, skin, and prostate cancers [77,80,81]. Together with CSCs, the epithelial–mesenchymal transition (EMT) is responsible for the metastatic propensity of cancer; in fact, it is reported that EMT cells show stem cell-like facets [82,83]. The Wnt/β-catenin signaling pathway is considered to be a critical inducer of the EMT process and is important in maintaining cancer stem cell properties [84], and β-catenin is the main mediator for the Wnt signaling from the cytoplasm through the nucleus [27,85,86]. Different studies show that defective functions in the Wnt/β-catenin pathway are the key oncogene stimulus in 90% of patients affected by colon cancer and, coincidently, this pathway has a principal role in CSCs maintenance in CRC patients [76,87].

MicroRNAs (miRs) are endogenously small, noncoding RNAs that can post-transcriptionally regulate gene expression and seem to play an important role in maintaining normal cellular functions [88]. Studies have shown that the miR-200 family plays a significant role in the inhibition of the proliferation and metastases potential of CSCs and EMT phenomenon by suppressing Wnt/catenin signaling [89]. One recent study showed that zerumbone could reverse EMT to the mesenchymal–epithelial transition (MET) through the upregulation of miR-200c by decreasing β-catenin expression in CRC HCT-116 and SW-48 cell lines and by inhibiting the transcription of genes involved in EMT and CSCs [90]. As a result, CRC HCT-116 and SW-48 cell lines showed reduced cell viability after zerumbone treatment.

Another study demonstrated that ZD 2-1, a mixture of ZD 2, novel zingerone derivative, and zingerone, significantly inhibited the TGF-β1 induced an increase in migration and invasion in SNU182 hepatocellular carcinoma cells when the concentration of ZD 2-1 reached 40 μM [18]. In particular, ZD 2-1 inhibited nuclear translocation of NF-kB and activation of p42/44 MAPK/AP1 signaling pathways in the TGF-β1 induced EMT, probably by inhibiting activation of MMP-2/9 and p42/44 MAPK [87].

In another experiment, 6-shogaol was found to interfere with the Notch pathway, which is known to be actively involved in the self-renewal of CSCs. The treatment of MCF-7 and MDA-MB-231 breast cancer lines, both in monolayer and 3D spheroids configuration, with 25 μM of 6-shogaol, reduced the cleavage of Notch1 in a time-dependent manner, and consequently, decreased the Notch targets (Hes1 and Cyclin D1), in this way interfering with the stem cell self-renewal pathway [13].

Additionally, the anti-tumor effects of [10]-gingerol in vivo was validated by using metastatic 4T1Br4 tumor-bearing mice [91]. Control mice over the subsequent 14 days of treatment showed weight loss, indicative of cachexia typically observed in mice with a high tumor burden. In contrast, mice from the [10]-gingerol (10 mg/kg) group gained some weight. The results indicated a significantly lower incidence of mice with brain lesions in the [10]-gingerol-treated group (1/13) compared to controls (7/13) [91]. Moreover, [10]-gingerol reduced spontaneous lung and bone metastatic burden. In addition, [10]-gingerol

was well-tolerated in vivo, induced a marked increase in caspase-3 activation, and inhibited orthotopic tumor growth in a syngeneic mouse model of spontaneous breast cancer metastasis. Importantly, by using both spontaneous and experimental metastasis assays, it was evidenced that [10]-gingerol significantly inhibited metastasis of multiple organs including lung, bone, and brain.

7. Ginger and Multidrug Resistance

Multidrug resistance (MDR) mechanisms are associated with increased expression of the P-glycoprotein (Pgp) or increased cellular metabolism of drug detoxifying proteins, such as glutathione-S-transferase (GST), that are correlated with increased resistance to apoptosis [24,92]. Additionally, multidrug resistance-associated protein 1 (MRP1), involved in the transport of many antitumor agents, is overexpressed in many chemoresistant cancer types including gastric cancer, neuroblastoma, and prostate cancer [93,94].

Recent research found that GST and MRP1 protein expression in the docetaxel-resistant human prostate (PC3R) cancer cell line was higher than in the docetaxel-sensitive human prostate (PC3) cancer cell line [95]. The results showed that 6-gingerol, 10-gingerol, 6-shogaol, and 10-shogaol inhibited the proliferation of PC3R cells through the downregulation of MRP1 and GST-protein expression [95]. Another study showed that a combined therapy of 6-gingerol with doxorubicin (doxo) could enhance the efficacy of doxo-based regiments in the treatment of Pgp-mediated MDR tumor with no severe side effects [12]. 6-gingerol in combination with doxo produced a significant increase of doxo accumulation (up to 44%) with the concentration of ginger at 10 and 20 μM in combination with doxo 2, 4, and 8 μM within the doxo-resistant human uterus sarcoma cell line MES-SA/Dx5. Additionally, the increase in GSH production was significant (up to 13%) at a higher (20 μM) 6-gingerol concentration [12]. So, on one hand, 6-gingerol could act as chemosensitizer inhibiting Pgp activity and, on the other hand, at high concentrations, it could have an anti-oxidative capacity that could be useful to protect MDR-negative normal cells against the damage caused by the generation of free radicals during anticancer treatment while its extrusion from resistant cells via Pgp could reduce the protective effects of cells increasing doxo sensitivity. Combined treatment of ginger oil with Methotrexate (MTX) increased the cytotoxic effect of MTX by 1.54-fold for the CCRF-CEM, T-cell Acute Lymphoblastic Leukemia (T-ALL) malignant cell line and 2.3-fold for Nalm-6 (B-ALL) cells, while the cytotoxic activity of this herbal extract in normal mononuclear cells was negligible [11]. Additionally, 11 out of 12 patient samples showed 1.2–16.5% increased apoptosis compared with the untreated samples. It was also shown that the more resistant cells were to the chemotherapy drug, the more sensitive they were to the medicinal herb. These data introduced ginger as a promising candidate for improved combination therapies in ALL, especially for those patients who show resistance to chemotherapy [11].

8. Ginger Enhanced Bioavailability and Combined Treatment

Low bioavailability alongside the poor solubility of ginger derivatives hinders their clinical application, probably due to poor absorption, hydrophobicity, extreme instability, and rapid metabolism, with concomitant elimination [96,97]. Recently, nanotechnologies (polymer nanoparticles or micelles, liposomes, inorganic nanoparticles, and nano-emulsions) have shown a huge advantage in enhancing bioavailability of these compounds as well as their oral absorption, reducing medicinal herb doses and toxicity, thereby improving the target ability and therapeutic effects [98,99]. Comparatively, self-assembled micelles could provide several advantages to drug delivery systems because of their high drug loading capacity, low dose of formulation required, and long circulation time [100,101]. In the recent study, the polyethylene glycol (PEG) derivative of linoleic acid (mPEG2K-LA) was first employed as a material for forming micelles to encapsulate 6-shogaol and enhance its solubility [102]. The formulated 6-shogaol loaded micelles (SMs) significantly slowed the drug release in stimulated media of the gastrointestinal tract and increased the sensitivity of tumor cells to the prototype drug. A high drug encapsulation of 80% was achieved under

a drug loading capacity of 7%, which greatly enhanced the 6-shogaol delivery efficiency versus general oral delivery systems. Therefore, SMs show a slower release rate than the free 6-shogaol [102]. More importantly, the in vitro cytotoxic effect of SMs in HepG2 cells is significantly higher than free 6-shogaol [102]. In addition, SMs showed enhanced oral bioavailability and liver and brain distribution compared to free 6-shogaol. The in vivo liver protection study in mice also demonstrated that SMs markedly reduced the activities of serum AST, ALT, and liver MDA levels, while they remarkably increased the antioxidant activities (GSH-Px, T-SOD). Therefore, the novel SMs are expected to serve as a promising carrier for 6-shogaol to enhance its cancer treatment and hepatoprotection [102].

Because radiotherapy is one of the main treatment options in head and neck cancer, 6-shogaol was combined with irradiation to evaluate a possible radiosensitizing effect [16]. The results of this study showed that 6-shogaol enhanced the effect of irradiation on SCC25, CAL27, two squamous cell carcinoma cell lines of the tongue and FaDu, and a squamous cell carcinoma cell line of the pharynx in vitro. Cell viability assays showed that irradiation in combination with 6-shogaol lead to a stronger growth inhibition than each treatment method alone. Experiments demonstrated a synergistic effect of 6-shogaol and irradiation, therefore a radiosensitizing capability of 6-shogaol can be supposed [16]. A recent study described a nanovector made from ginger-derived lipids that can serve as a delivery platform for the therapeutic agent doxorubicin (doxo) to treat colon cancer [28]. The nanoparticles from ginger were created and their lipids were reassembled into ginger-derived nanovectors (GDNVs). A subsequent characterization showed that GDNVs are efficiently taken up by colon cancer cells. Modified GDNVs conjugated with the targeting ligand folic acid-mediated targeted delivery of doxo to Colon-26 tumors in a xenograft tumor model in vivo and enhanced the chemotherapeutic inhibition of tumor growth compared with free drug [28]. Such delivery vehicles have enhanced the permeability and retention effect that allowed drugs to reach tumors more passively through leaky vasculatures surrounding the mass. The result of this study demonstrated that GDNVs loaded with doxo successfully inhibited tumor growth in a Colon-26 xenograft tumor model [28].

Additionally, the studies reported the effects of combined treatment (ginger and gelam honey), which downregulate the gene expressions of Akt, mTOR, Raptor, Rictor, β-catenin, Gsk3β, Tcf4, and cyclin D1 while cytochrome C and caspase 3 genes were shown to be upregulated in HT29 colon cancer cells [103]. Furthermore, an extract mixture of turmeric, ginger, and garlic induced apoptosis in MCF-7 and ZR-75 breast cancer cell lines [104]. A combined treatment with NE mix-tamoxifen caused the extension of apoptosis indicating a potential role of the NE mix in sensitizing the ER-positive breast cancer cells towards tamoxifen. Moreover, a combined treatment with NE mix-tamoxifen altered the expression of apoptotic markers (p53 and Caspase 9) leading to apoptosis in breast cancer cell lines.

So, nanotechnologies and combined treatment seem to increase the efficiency of ginger derivatives therapeutic effects by increasing their bioavailability.

9. Conclusions and Future Perspectives

Nowadays, available drugs for treating cancer are often toxic, expensive, and little effective. Ginger derivatives possess high potential chemopreventive properties such as cell cycle arrest, increased cellular death (apoptosis, autophagy and autosis), as well as redox homeostasis unbalance. Furthermore, they inhibit angiogenesis, CSCs formation, and the EMT process. Therefore, this natural compound directly and indirectly influences tumor cell survival and inhibits invasion and metastasis processes, without significant toxic effects on normal cells [18,26]. Additionally, ginger enhances the therapeutic efficacy of the currently available anti-cancer drugs and represents a good adjuvant to certain phytochemical compounds including turmeric, garlic, and gelam honey [103,104]. A very important moment in ginger derivatives' chemopreventive properties is that they do not cause side effects, but on the contrary, they ease the side effects provoked by other cancer treatments, like radio- and chemotherapy, giving ginger a considerable advantage in being considered

a chemopreventive natural compound [105,106]. However, most of the known activities of ginger components have been studied only in in vitro and in vivo studies, except for a few clinical studies in human subjects. Therefore, more substantial and well-controlled clinical human studies are needed to illustrate its efficacy as an anticancer agent, since it is a safe and encouraging alternative. The recent development of nanotechnologies (polymer nanoparticles or micelles, liposomes, inorganic nanoparticles, and nano-emulsions) provides a chance to improve oral absorption, bioavailability of ginger, while also improving the target ability and its therapeutic effects [28,102]. In summary, ginger derivatives have various health effects and therapeutic properties; nevertheless, their biological applications are limited due to their hydrophobic nature. The low aqueous solubility of this compound seems to be the major obstacle for its lab-to-clinic development as a drug; therefore, it appears to be necessary to use advanced extraction methods to improve its bioavailability.

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Abbreviations

ALT	alanine aminotransferase
AP-1	activator protein 1
AST	aspartate aminotransferase
ATPase	adenosine triphosphatase
B-ALL	B-cell Acute Lymphoblastic Leukemia
Bcl-2	B-cell lymphoma 2
CAM	chorioallantoic membrane
Caspases	cysteine-aspartate proteases
CAT	catalase
CDK	cyclin-dependent kinases
CFEZO	crude flavonoid extracts from Z. officinale
CKI	cyclin-dependent kinases inhibitor
COX-2	cyclooxygenase-2
CRC	colorectal carcinoma
CSCs	cancer stem cells
CuZn-SOD Cu-Zn	superoxide dismutase
CYP-P450	cytochrome P450
DMBA	0.5% 7,12-dimethylbenz[a]anthracene
DNA	deoxyribonucleic acid
DNPH	dinitrophenylhydrazone
ECOG	Eastern Cooperative Oncology Group
EE	Etlingera elatior
EMT	epithelial-mesenchymal transition
eNO	endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
FAs	focal adhesions
GAE	aqueous extract of ginger
GDNVs	ginger-derived nanovectors
GR	glutathione reductase
GSH	Glutathione
GST	glutathione-S-transferase
HBP	hamster buccal pouch
HNSCC	head and neck squamous cell carcinoma
HUVECs	human umbilical vein endothelial cells
IAP	inhibitor of apoptosis
IL-8	interleukin 8

LC3-II	light chain3-II
MAPK	Mitogen-Activated Protein Kinase
MDA	malondialdehyde
MDR	multidrug resistance
MET	esenchymal–epithelial transition
miRs	microRNA
MMP	Matrix metalloproteinase
MRP1	multidrug resistance-associated protein 1
mTOR	the mechanistic target of rapamycin
MTX	methotrexate
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nox	NADPH oxidases
NSCLC	non-small-cell lung cancer
PEG	Polyethylene Glycol
Pgp	P-glycoprotein
PI3K	phosphatidylinositol-3-kinase
RNA	ribonucleic acid
ROS	reactive oxygen species
SCID	severe combined immune deficient
SDGE	Steam Distilled Extract of Ginger
SMs	6-shogaol loaded micelles
SSHE	extract of Syussai ginger
STAT3	signal transducer and activator of transcription 3
T-ALL	T-cell Acute Lymphoblastic Leukemia
TNBC	triple negative breast cancer
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
USP14	ubiquitin-specific peptidase 14
VEGF	vascular endothelial growth factor
XO	xanthine oxidase
ZOME	Methanolic extract of Zingiber officinale rhizome

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Neovascularization is a key feature of liver fibrosis progression: anti-angiogenesis as an innovative way of liver fibrosis treatment

Mariia Zadorozhna, Sante Di Gioia, Massimo Conese & Domenica Mangieri

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MINI REVIEW ARTICLE

Neovascularization is a key feature of liver fibrosis progression: anti-angiogenesis as an innovative way of liver fibrosis treatment

Mariia Zadorozhna[1] · Sante Di Gioia[1] · Massimo Conese[1] · Domenica Mangieri[1]

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Abstract
Liver fibrosis affects over 100 million people in the world; it represents a multifactorial, fibro-inflammatory disorder characterized by exacerbated production of extracellular matrix with consequent aberration of hepatic tissue. The aetiology of this disease is very complex and seems to involve a broad spectrum of factors including the lifestyle, environment factors, genes and epigenetic changes. More evidences indicate that angiogenesis, a process consisting in the formation of new blood vessels from pre-existing vessels, plays a crucial role in the progression of liver fibrosis. Central to the pathogenesis of liver fibrosis is the hepatic stellate cells (HSCs) which represent a crossroad among inflammation, fibrosis and angiogenesis. Quiescent HSCs can be stimulated by a host of growth factors, pro-inflammatory mediators produced by damaged resident liver cell types, as well as by hypoxia, contributing to neoangiogenesis, which in turn can be a bridge between acute and chronic inflammation. As matter of fact, studies demonstrated that neutralization of vascular endothelial growth factor as well as other proangiogenic agents can attenuate the progression of liver fibrosis. With this review, our intent is to discuss the cause and the role of angiogenesis in liver fibrosis focusing on the current knowledge about the impact of anti-angiogenetic therapies in this pathology.

Keywords Liver fibrosis · Angiogenesis · Anti-angiogenic drugs · Liver information · Liver fibrosis regression

Introduction

Genetic, environmental and lifestyle factors (e.g. alcohol abuse), mechanotransduction signal pathway and viral infections can contribute in onset and progression of liver fibrosis (LF) [1–6]. Histologically, this disorder can be classified as a chronic fibro-inflammatory condition characterized by an excessive deposition of extracellular matrix (ECM) proteins including collagen fibers (I, III, and IV) [7–9]. Clinically, portal hypertension can be a key feature in patients suffering from severe form of LF [10, 11]. Evidence from a number of studies demonstrates that angiogenesis, the formation of new blood vessels from pre-existing vasculature, plays a crucial role in the progression of this complex disease [12–15]. It is well known that inflammation and hypoxia are two elements that strongly promote neovascularization [16–18]. Interestingly, both phenomena can be considered as markers of LF [19, 20]; thus, it is reasonable that angiogenesis takes place during hepatic fibrogenesis [12, 13]. Consequently, it is conceivable that anti-angiogenic approaches could represent a useful tool in the treatment of LF. The present review will describe the general aspects of the pathogenesis of LF, focusing on the link between hepatic fibrogenesis and angiogenesis. Meanwhile, selective strategies targeting angiogenesis for the preservation of the hepatic tissue will be introduced.

Mariia Zadorozhna and Massimo Conese have contributed equally to this work.

✉ Domenica Mangieri
domenica.mangieri@unifg.it

Mariia Zadorozhna
mariia.zadorozhna@unifg.it

Sante Di Gioia
sante.digioia@unifg.it

Massimo Conese
massimo.conese@unifg.it

[1] Department of Medical and Surgical Sciences, University of Foggia, Via Pinto 1, 71122 Foggia, Italy

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The pathogenesis of liver fibrosis

As aforementioned, histologically, fibrotic hepatic parenchyma is characterised by chronic inflammation and exacerbated production of ECM molecules with consequent abnormality in the liver tissue [7, 8]. The inflammatory foci are comprised of lymphocytes, plasma cells, monocytes/macrophages (LY6C^{hi} phenotype) as well as granulocytes [21, 22]. All these inflammatory components indirectly participate in the process of fibrogenesis by producing soluble/paracrine signals including cytokines, chemotactic molecules, fibrogenic agents [23, 24]. Also, in the chronic hepatic injuries, cholangiocytes, hepatocytes, liver sinusoidal endothelial cells (LSECs) and non-sinusoidal endothelial cells (ECs), together with resident Kupffer cells secrete various sclerotic stimuli such as transforming growth factor- β (TGF-β, the "master mediator" of many fibrotic disorders), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) [25]. Figure 1 depicts the many cell types and molecular effectors involved in LF, leading to the activation of HSCs. Generally, the primary effectors of fibrogenesis are resident fibroblasts, myofibroblasts [26, 27], and their bone marrow-derived circulating precursors namely fibrocytes [28, 29]. In damaged liver, activated hepatic stellate cells (HSCs) are mainly

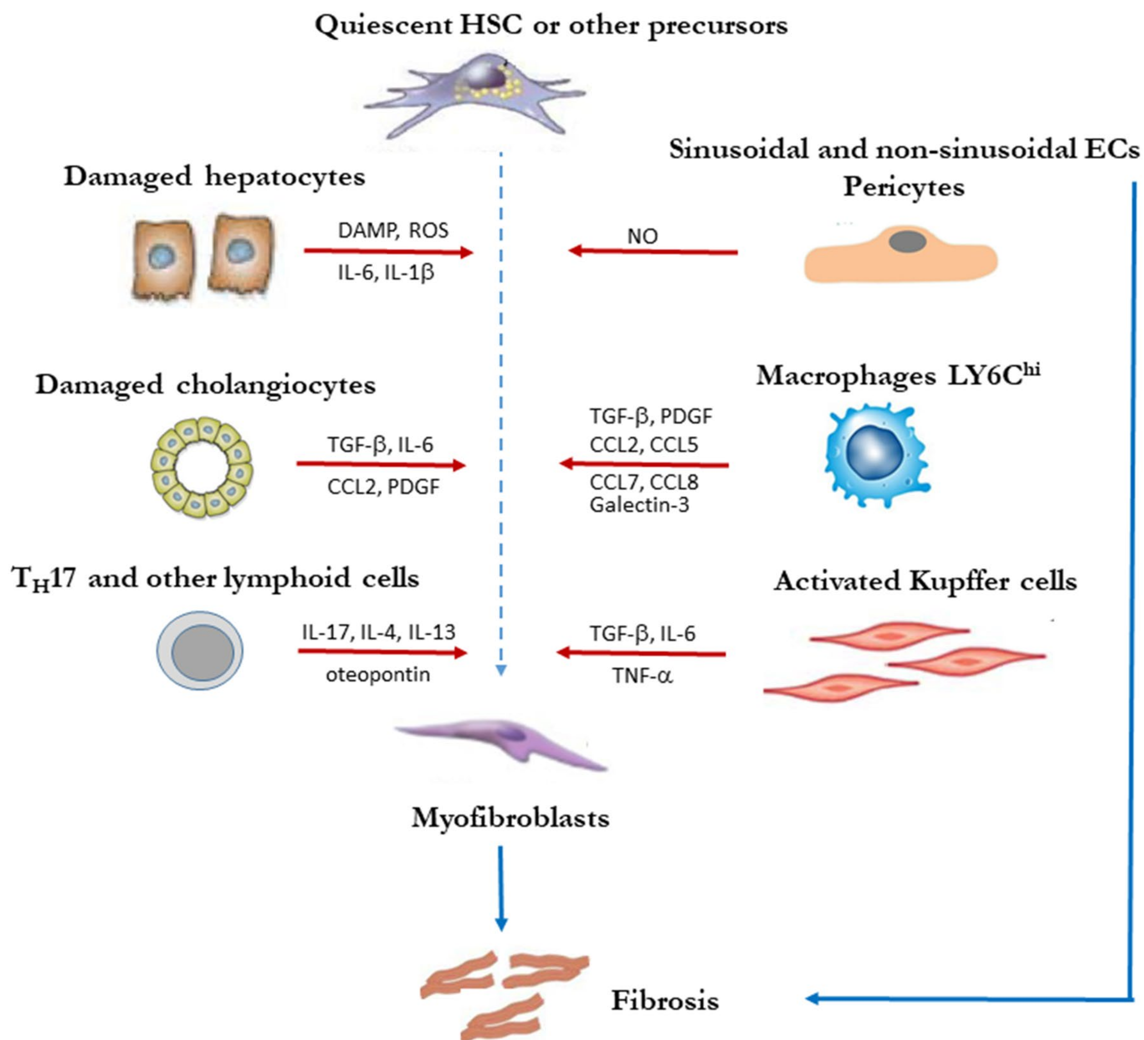

Fig. 1 During liver injury, quiescent HSCs or other precursors (e.g. bone marrow-derived fibrocytes, portal fibroblasts, hepatocytes in the epithelial-mesenchymal transition) are activated by various cell types resident in the liver, including hepatocytes, cholangiocytes, sinusoidal and non-sinusoidal endothelial cells, pericytes, macrophages LY6C^{hi}, Kupffer cells, as well as Th17 T cells and other lymphoid cells. All these cell types secrete pro-fibrogenic mediators that ultimately activate HSCs or other precursors that eventually transform into myofibroblasts and operate to deposit ECM. *CCL* CC chemokine ligands, *DAMP* danger associated molecular pattern, *IL* interleukin, *NO* nitric oxide, *PDGF* platelet-derived growth factor, *ROS* reactive oxygen species, *TGF-β* transforming growth factor-β, *TNF-α* tumor necrosis factor-α

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responsible of fibrogenesis in at least 2 ways: on one hand they produce ECM, on the other hand impede ECM degradation by secreting proteases inhibitors including endogenous tissue inhibitors of metalloproteinases (TIMPs) [30, 31]. There is a lot of evidence showing that epithelial-mesenchymal-transition (EMT) has also a great importance in fibrotic lesions. Accordingly, hepatocytes as well as ECs/LSECs can undergo a process of epithelial-(endothelial)-mesenchymal transition (EMT) through autocrine/paracrine signals mediated, in part, by TGF-β [32]. Additionally, recent work discovered that pericytes, are also involved in the process of fibrogenesis [33–35]. In fact, these mural cells seem to have the capability to detach from basement membrane surrounding hepatic capillary to accumulate within the injured hepatic tissues, where they undergo phenotypic transformation into ECM-producing myofibroblasts [33–35]. As a consequence, a wide range of cells, growth factors and other stimuli are engaged in the liver fibrogenesis [33–35].

The link between angiogenesis and liver fibrosis

Angiogenesis is a growth factor-dependent phenomenon taking place during all stage of the human development; during adult life, at least in healthy conditions, it happens only in certain circumstances, for example during pregnancy and menstrual cycle [36, 37]. By contrast, experimental and clinical evidences indicate that angiogenesis accelerates the progression of many disorders such as cancer growth and metastasis, rheumatoid arthritis, diabetic retinopathy and other complex diseases including LF [12, 38–42]. It is well known that inflammation and hypoxia are crucial elements in induction of neovascularization. As previously specified, hepatic tissue affected by fibrosis, shows permanent inflammation and low oxygen level, offering a prototypical microenvironment for neovascularization [43, 44]. In detail, accumulation of ECM in liver parenchyma is a main cause of hypoxia, which in turn, stabilizes the dimeric transcription factor “hypoxia-inducible factor” (HIF) [45]. HIF regulates the transcription of an array of genes including those controlling angiogenesis such VEGF, PDGF-B, matrix metalloproteinases (MMPs) as well as TIMPs [46–49]. As matter of fact, hypoxic areas co-localize with those of an increased microvessel density (MVD), fibrous septa and inflammatory foci [48, 50, 51]. In addition, hypoxia further stimulates the infiltration of inflammatory cells [52], which, in turn, contribute to angiogenesis and fibrotic phenomena [53]. In conclusion, in injured liver, hypoxia, angiogenesis, chronic inflammation and fibrosis drive each other following an activated loop, and synergistically exacerbate the severity of the LF (Fig. 2) [15, 16, 54].

To incite neoangiogenesis, VEGF binds to its receptors VEGFRs stimulating the formation of new functional vessels (Fig. 3). By the signals generated by bound VEGFRs, VEGF is the leading regulator of ECs/LSECs activity during all steps of angiogenesis [55]. In LF, VEGF is, in part, produced by ECs/LSECs themselves suggesting an autocrine action of this signal pathway; but damaged hepatocytes and activated HSCs seem to be the principal sources of this relevant growth factor [56]. The latter evidence highlights the crucial role of HSCs in LF because they constitute a crossroad among inflammation, fibrosis and angiogenesis (Fig. 4) [12, 16, 57, 58]. PDGF-B, principally produced by ECs/LSECs, acts during vessel stabilization, orchestrating the formation/maturation of vascular tube and its coverage through the recruitment of PDGFRs

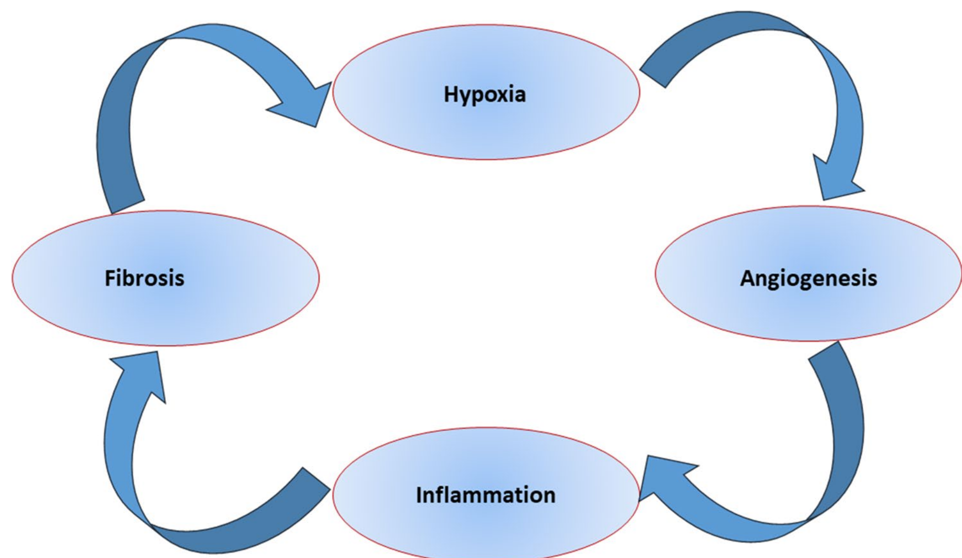

Fig. 2 Link among hypoxia, angiogenesis, inflammation and liver fibrosis. Hypoxia, angiogenesis, inflammation and fibrosis drive each other activating a pathological loop in liver

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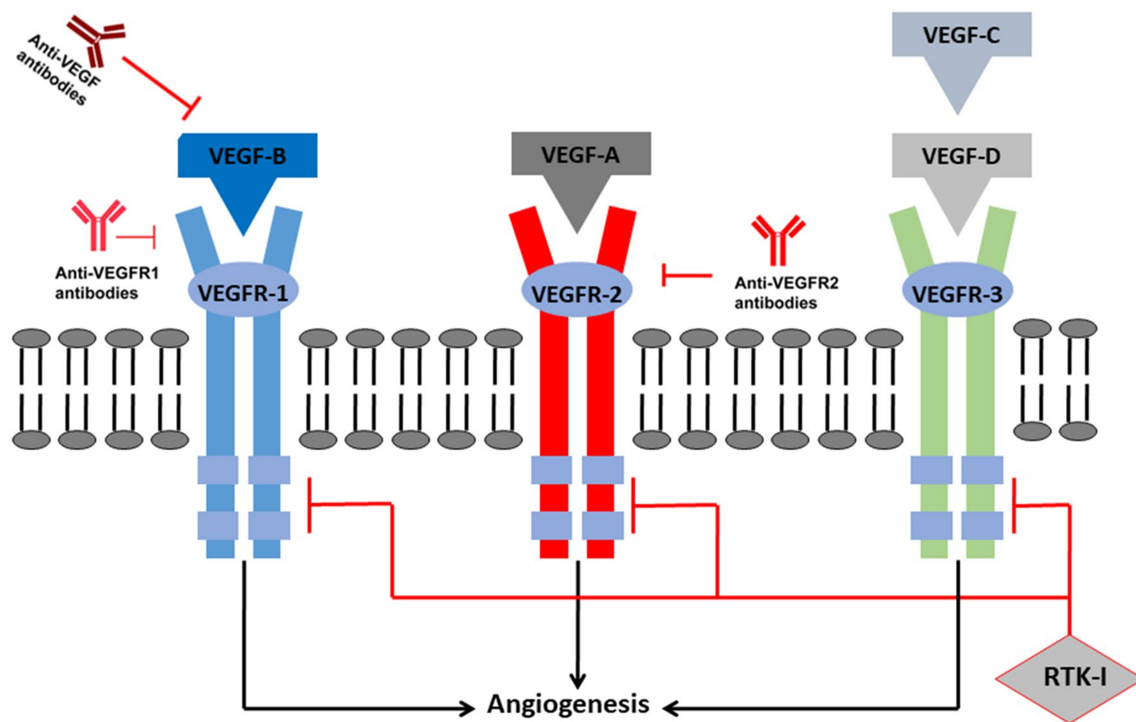

Fig. 3 The VEGF/VEGFR signalling axis, its contribution to angiogenesis and treatment modalities interfering with its activity. Binding of VEGF ligands to their cognate receptors (VEGFRs) leads to receptor dimerization and autophosphorylation triggering a downstream intracellular phosphorylation cascade. Monoclonal antibodies target VEGFs, preventing its binding to VEGFRs, while monoclonal antibodies targeting VEGFRs prevent the binding of VEGFs, resulting in the inhibition of VEGFR signalling. The treatment of receptor tyrosine kinase inhibitors (RTK-Is) inhibits the activation of VEGF/VEGFR signalling

positive pericytes/HSCs [57]. FGF, through an autocrine loop, is involved in LF angiogenesis not only inducing the activation of ECs/LSECs, but also, increasing HSC proliferation and recruitment [12]. Ang-2 is acutely released from activated ECs/LSECs, upon stimulation with inflammatory cytokines, proangiogenic factors, and hypoxia and competitively inhibits the binding of Ang-1 to Tie-2 [59] that, instead, serves to maintain survival and quiescence of endothelium [60]. Inflammatory cells also secrete a plethora of angiogenic factors (VEGF, PIGF, PDGF, FGF, Angiopoietins, TGF-β, etc.) [12, 61–67]. For example, both infiltrating macrophages and resident Kupffer cells, once activated, contribute to angiogenesis releasing reactive oxygen species (ROS), nitric oxide (NO), tumor necrosis factor- α (TNF-α) and other angiocrine molecules [12]. As above cited, in drastic circumstances, LF is complicated by portal hypertension (PHT) accompanied by severe hepatic structural disorder correlated to diffuse fibrosis [68]. Angiogenesis also participates in the pathogenesis of PHT, in part modulating HSCs activation and, on the other hand, provoking the formation of portal-veins collaterals [69]. Cellular molecules involved in promoting angiogenesis and their roles in LF are listed in Table 1 [57, 70–75].

Anti-angiogenesis approaches slow down LF

Considering the indisputable importance of neovascularization in LF progression, it is plausible that blocking angiogenesis may offer a method to attenuate the aberration of hepatic tissue or prevent more serious damage including cirrhosis [14, 76]. For this reason, some anti-angiogenic strategies including natural compounds are currently under investigation [77]. Since VEGF is the most efficient proangiogenic factor, generally most anti-angiogenic therapies have been focused on blocking the VEGF signal pathway [76, 78] (Fig. 3). Bevacizumab, a humanized monoclonal antibody neutralizing VEGF-A [72, 79, 80], in combination to other drugs, is currently used to treat different kinds of tumors [74, 81, 82]. It also shows a strong anti-fibrotic effect in human Tenon's fibrosis [83]. High VEGF-A levels in the aqueous humor of patients with nonneovascular glaucoma have been reported [84], and this increase may contribute to post-operative inflammation and fibrosis. Since Tenon's fibroblasts have been shown to express VEGF-A receptors [84], these findings highlight once more the intimate relationship between angiogenesis and fibrosis, as it occurs in LF. An interesting study conducted by Huang et al. [85], showed that bevacizumab alleviates LF in vivo by

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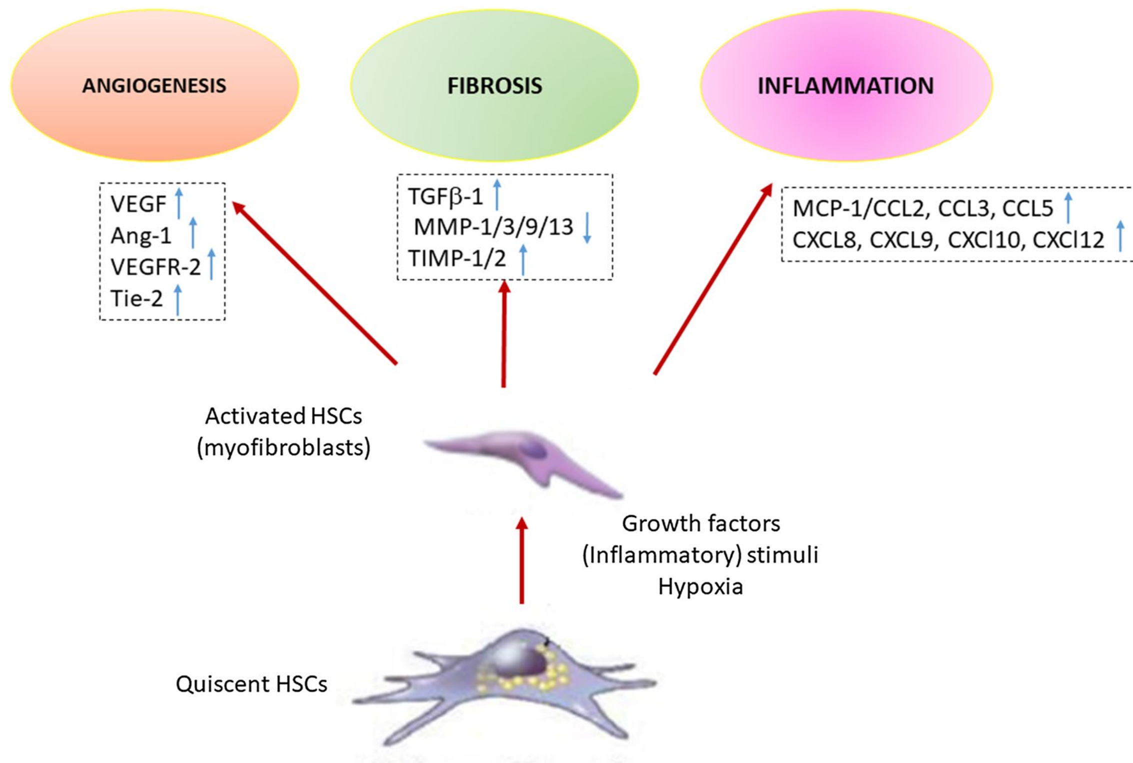

Fig. 4 Schematic model of HSCs activation. Quiescent HSCs are activated, during lung injury, by a host of factors, including hypoxia, inflammatory stimuli and growth factors produced by liver cells, such as hepatocytes and endothelial cells. HSCs transform into myofibroblasts and contribute to angiogenesis, fibrosis and inflammation. Once activated, HSCs act as proangiogenic cells and may respond to stimuli such as hypoxia through the increase of VEGF, Ang-1, and their related receptors VEGFR-2 and Tie-2. Activated HSCs are the prime downstream effectors of excess ECM deposition and they also produce the fibrogenic cytokine TGF-β. Moreover, fibrolysis is compromised, e.g. by an increased synthesis of TIMPs and a decreased production of fibrolytic MMPs. Finally, activated HSCs contribute to inflammation in liver fibrosis by producing chemokines, including CC chemokines ligands (CCL2, CCL3, CCL5) and the CXC chemokines ligands (CXCL8, CXCl9, CXCL10, CXCL12). *MCP-1* monocyte chemoattractant protein-1

neutralizing VEGF produced by hepatocytes and by blocking HSCs activation. The effects of VEGFRs neutralizing antibodies such as anti-VEGFR1 and anti-VEGFR2 have also been explored [86, 87] (Fig. 3). Results show that the use of anti-VEGFR-2 antibody results more effective than the anti-VEGFR-1 antibody when used alone [87], although combined treatment with both antibodies gave some tissue improvement in LF [87]. Additionally, the use of the multiple receptor tyrosine kinase inhibitors such as sorafenib and sunitinib blocking PDGFR-β and VEGFRs signaling pathways is under investigation [88] (Fig. 3). Sorafenib attenuates LF by reducing HSCs proliferation/activation and inducing their apoptosis both in vitro and in vivo [88, 89]. Sunitinib also decreases LF, switching off inflammation, HSCs activation and angiogenesis [90, 91]. PDGF-B and its signaling pathway and cyclooxygenase-2 are also involved in HSCs activation [92, 93]. Gao at al demonstrated that the use of celecoxib (a cyclooxygenase-2 inhibitor) shows similar effect in vivo as those obtained with sorafenib [94]. It is implicit that enhancing the expression and the activation of the ECM proteases can also contribute to the resolution of fibrosis [95, 96]. In line with this idea, it has been shown that the decrease of LF is associated with increased expression of MMPs (MMP-2 and -14) as well as decreased expression of TIMP-1 and -2 in hepatic tissue [30, 97].

Along with the use of immunotarget therapy above listed, recently, the regenerative potential of stem cells is being exploited in fibrotic diseases. Accordingly, the injection of bone marrow-derived mesenchymal stem cells (BMSCs) including endothelial progenitor's cells (EPCs) seems to reduce the severity of LF by increasing the degradation of ECM by means of proteases/MMPs [98, 99]. In fact, experimental evidences showed that EPCs transplantation was shown to effectively promote the remodelling of damaged liver tissues in a dimethylnitrosamine (DMN) rat liver fibrosis model [100, 101].

Other approaches to slow down LF

Additionally, studies have demonstrated that LF may be prevented or reversed by bioactive food components and natural products, including fumagillin analogue (TNP-470),

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Table 1 Molecules involved in angiogenesis and their role in LF cited in this review

Angiogenic factor	Actions during angiogenesis	Role in angiogenesis in LF	References
VEGF	• Promotes endothelial cell survival and homeostasis • Promotes endothelial cell detachment from the basement membrane • VEGF and Notch co-operate in an integrated intercellular feedback that functions as a ''branching pattern generator''	Produced by damaged hepatocytes and activated HSC → capillarization of sinusoids	[70]
PDGF-B	Recruitment of pericytes	Produced by ECs/LSECs this factor stimulates HSC proliferation, differentiation, and migration, as well as transforms HSC into myofibroblasts	[57]
TGF-β	Stimulates mural cell induction, differentiation, proliferation, and migration and promotes production of extracellular matrix	Release of TGF-β by necrotic hepatocytes during liver damage is one of the first signals to activate adjacent quiescent HSC → trans-differentiation into myofibroblasts	[71]
FGF	This factor is mitogenic for endothelial cells and increases the expression of VEGF	Induces the activation of ECs/LSECs, and increases HSC proliferation and recruitment	[72, 73]
ANG1 and Tie-2	ANG1, produced by mural cells, activates its endothelial receptor Tie-2 ANG1 stabilizes vessels, promotes pericyte adhesion, and makes them leak resistant by tightening endothelial junctions	Autocrine ANG1 promotes HSC/myofibroblast migration	[70]
EGF and TGF-α	They are mitogenic for endothelial cells and increase angiogenesis in in vivo model	Hepatocyte-derived EGF induces HSC migration Autocrine TGF-α is involved in transformation into myofibroblasts	[74, 75]

astaxanthin, curcumin, blueberry, silymarin, vitamins (C, D, E), resveratrol, quercetin, coffee and green tea extracts [102–104]. Generally, the anti-fibrotic effect of all these natural compounds seems to be mainly attributed not only to their antioxidant and anti-inflammatory features but also to their ability to revert the activated forms of HSCs in a more quiescent phenotype [102].

Current challenges and future directions

Inflammation, fibrosis and angiogenesis are strictly intertwined during the progression of chronic liver diseases (CLDs), including chronic viral hepatitis, PTH, non-alcoholic and alcoholic liver diseases. This brings to the notion that a wealth of cellular and molecular mechanisms are implicated in liver fibrosis and angiogenesis. Interactions among hepatocytes, HSCs, Kupffer cells, and endothelial cells have been described, with HSCs representing a crossroad at the interaction between inflammation, angiogenesis, and fibrosis. Angiogenic factors, including VEGF, PDGF, FGF, Ang-2, EGF, and various cytokines, are important mediators of angiogenesis in fibrosis associated with CLDs. Besides these factors, metabolic abnormalities, including adipokines, may dysregulate angiogenesis, and hence influence inflammation and fibrosis. Moreover, it has also been shown that endoplasmic reticulum stress and related unfolded protein response, and neuropilins are involved in liver angiogenesis and fibrosis [12]. Given the plethora of cellular and molecular mechanisms, a better appraisal of this complexity may be caught by three-dimensional (3D) models that can recapitulate liver architecture and interactions among different cell types [105, 106]. Indeed, one obstacle in the development of efficient therapies is the lack of robust and representative in vitro models of human liver fibrosis through which novel drugs can be tested. Currently used animal models are not useful for dissecting the relative role of each component since the predictive value for human physiologic responses in terms of pharmacokinetics and pharmacodynamics is sometimes poor. Moreover, they are not suitable for large scale screening of antifibrotic compounds. The main 3D models that are being used and implemented include cocultures of hepatocytes and HSCs, achieved by insert cultures, spheroids (presenting many cell types), or liver tissue cultures. More advanced techniques are bioprinting and microfabricated microfluidic devices to provide a constant flow of oxygen and fresh nutrients and remove the metabolic waste generated (as replacement of bile canaliculi). Finally, organotypic models, such as precision cut liver slices and decellularised 3D scaffolds, will offer more opportunities to test novel drugs in a context maintaining the intact hepatic architecture and cellular heterogeneity. Thus far, the main focus of the field has been on the maintenance of functional hepatocytes for prolonged culture periods;

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incorporation of non-parenchymal cells (such as endothelial cells, Kupffer cells, and HSCs) will allow the use of these culture systems for in vitro fibrosis studies [105]. In order to gain higher number of cells and make sustainable these models, stem cells are a suitable source of different cell types. Many stem cell types, including liver progenitor/stem cells, extra-hepatic biliary tree stem cells, embryonic stem cells as well as induced pluripotent stem cells (iPSCs) have been reported to generate hepatocyte-like cells [107] and cholangiocytes [108, 109], and more recently LSECs and HSCs [110]. Further studies should determine whether these cell types are fully functional and can reconstitute organotypic models. Another essential feature of these models will be the inclusion of stiffer materials mimicking the deposition of collagen that is a feature of liver fibrosis. Depending on the hardness of the substrates used, i.e. soft versus stiff, the quiescent phenotype of HSCs will be maintained, or they will transform into activated myofibroblasts [5, 106]. Recently, a novel 3D organotypic liver models comprised of hepatocytes, LSECs, HSCs, Kupffer cells, and the Space of Disse mimic demonstrated how a mechanical gradient resulted in transitioning phenotypes in hepatic cells and cause varying profiles of fibrotic markers [111]. Thus, mechanotransduction and biomechanics are parameters that should be envisioned as essential in constructing these models. These advanced in vitro models have been used for testing drug induced liver injury, determined by alcohol or medications, in the developmental phase of pharmaceuticals [105, 112] or in the evaluation of drugs already in clinical trials [113]. In addition to inhibitors of angiogenesis, that could result in unspecific effects, genetic tools may target profibrotic and proangiogenetic genes with an unprecedented precision. Small interfering RNAs and antisense oligonucleotides have been vehicled by nanocarriers (lipoplexes and nanoparticles) that are preferentially engulfed by nonparenchymal cells, prominently HSCs and myofibroblasts. The target genes to be downregulated include TGFβ-1, TGFβ receptors, osteopontin, integrins, and chemokine receptors [58]. Complex 3D and organotypic models are also essential in finding novel noninvasive markers of angiogenesis in liver fibrosis. Histological follow-up does not have the power to reliably detect antifibrotic drug effects in the short term. Validated serum markers would measure the activity of angiogenesis and fibrogenesis and therefore enable the selection of patients likely to respond to antiangiogenic and antifibrotic therapies, and to detect responders to these therapies. Finally, these models could capture the inter-individual genetic and environmental variations, increasing the pace towards the personalised medicine approach [58], and will be paramount to design more precise and real-to-life clinical trials.

Relevant conclusion

Anti-angiogenic therapy for hepatic fibrosis resolution has received increasing attention in recent years. However, it is not possible to overlook the fact that the LF is a multifactorial disorder, and angiogenesis in only one of the phenomena that favours its genesis and progression. Moreover, the limited preclinical/clinical studies impede to know in detail any counterproductive effects of antiangiogenic therapies in this aberrant circumstance. Consequently, further large randomized studies need to be conducted before deducing that anti-angiogenic approaches can be used in the treatment of liver fibrosis.

Author contributions MD wrote and supervised manuscript; DGS assisted in the final preparation of the manuscript; ZM assisted manuscript preparation; CM wrote "Current Challenges and Future Directions" and supervised the final version of the manuscript.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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