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1 Anticancer potential of allicin: A review

Elena Catanzaro ^a, Donatella Canistro ^b, Valentina Pellicioni ^a, Fabio Vivarelli ^b, Carmela Fimognari
 ^{a*}

^a Dipartimento di Scienze per la Qualità della Vita, *Alma Mater Studiorum*-Università di Bologna,
 corso d'Augusto 237, 47921 Rimini, Italy. <u>elena.catanzaro2@unibo.it</u> (EC),
 valentina.pellicion2@unibo.it, carmela.fimognari@unibo.it (CF)

- ⁷ ^b Dipartimento di Farmacia e Biotecnologie, *Alma Mater Studiorum*-Università di Bologna, via Irnerio
- 8 48, 40126 Bologna, Italy. <u>donatella.canistro@unibo.it</u> (DC), <u>fabio.vivarelli3@unibo.it</u> (FV)
- 9 * Corresponding author:
- 10 Prof. Carmela Fimognari
- 11 Dipartimento di Scienze per la Qualità della Vita
- 12 Alma Mater Studiorum-Università di Bologna
- 13 corso d'Augusto 237, 47921 Rimini, Italy
- 14 Phone number: +39 0541 434658
- 15 E-mail address: carmela.fimognari@unibo.it

16 ABSTRACT

- 17 Phytochemicals have attracted attention in the oncological field because they are biologically
- 18 friendly and have relevant pharmacological activities. Thanks to the intense and unique spicy aroma,
- 19 garlic is one of the most used plants for cooking. Its consumption is correlated to health beneficial
- 20 effects towards several chronic diseases, such as cancer, mainly attributable to allicin, a bioactive
- 21 sulfur compound stored in different plant parts in a precursor form. The objective of this review is
- 22 to present and critically discuss the chemistry and biosynthesis of allicin, its pharmacokinetic profile,
- 23 its anticancer mechanisms and molecular targets, and its selectivity towards tumor cells. The
- 24 research carried out so far revealed that allicin suppresses the growth of different types of tumors.
- 25 In particular, it targets many signaling pathways associated with cancer development. Future
- 26 research directions are also outlined to further characterize this promising natural product.
- 27 Keywords: Allicin, Anticancer Drugs, Cancer, Cytotoxicity, Pharmacokinetics, Selectivity

28 Abbreviations: 5-FU, 5-fluorouracil; 6-MP, 6-mercaptopurine; 6-MPR, 6-MP-riboside; AFB1, 29 aflatoxin B1; AGE, aged garlic extract; AIF, apoptosis-inducing factor; AKT, protein kinase B; ALP, 30 alkaline phosphatase; ALT, alanine aminotransferase; AMS, allyl methyl sulfide; Apaf-1, apoptotic 31 protease activating factor-1; AST, aspartate aminotransferase; ATRA, all-trans retinoic acid; AUC, 32 area under the curve; Bak, Bcl-2 antagonist/killer; Bax, Bcl-2-like protein 4; bFGF, basic fibroblast 33 growth factor; BID, BH3 interacting domain death agonist; CAs, chromosomal aberrations; cdc2, 34 cyclin-dependent kinase 1; CLL, B-chronic lymphoblastic leukemia; CSC, cancer stem cells; CYP, 35 cytochrome P450; DIABLO, direct inhibitor of apoptosis-binding protein with low PI; DISC, death-36 inducing signaling complex; DX, dexamethasone; DOX, doxorubicin; EAC, Ehrlich ascites carcinoma; 37 EC, endothelial cells; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; Erb2, 38 human epidermal growth factor receptor 2; ERK, extracellular signal-regulated kinases; FADD, FAS-39 associated death domain; FASL, FAS ligand; FASR, FAS receptor; GBM, glioblastoma multiforme; 40 gGT, gamma glutamyl transferase; GSH, glutathione; GSSA, S-allylmercaptoglutathione; GSSG, 41 glutathione disulfide; Gy, gray; HCMV, human cytomegalovirus; HCV-ABxtl68, anti-Hepatitis C Virus 42 human monoclonal antibody; HDF, human dermal fibroblasts; HIF, hypoxia-inducible factor; HtrA2, 43 high-temperature requirement A2; IAPs, apoptosis inhibitor proteins; IC₅₀, half maximal inhibitory 44 concentration; IFN-β, interferon β; IkB, NF-kB inhibitory protein; IKK, IkB kinase; IL, interleukin; iNOS, 45 inducible nitric oxide synthase; i.p., intraperitoneal; i.v., intravenous; JNK, c-Jun N-terminal kinase; 46 LDH, lactate dehydrogenase; MAPKs, mitogen-activated protein kinases; MD, Mediterranean diet; 47 MGMT, O⁶-methylguanine-DNA methyltransferase; MMP matrix metalloprotease 9; MMS, methyl 48 methanesulfonate; mTOR, mechanistic target of rapamycin; NAC, N-acetylcysteine; NF-κB, nuclear 49 factor kappa-light-chain-enhancer of activated B cells; NK, natural killer; NRD, normal rat diet; Nrf2, 50 nuclear factor-erythroid factor 2-related factor 2; p62, sequestosome-1; PARP, Poly (ADP-ribose) 51 polymerase-1; PBMC, peripheral blood mononuclear cells; P-gp, P-glycoprotein; PI3K,

52 phosphoinositide-3-kinase; PIGF, placental growth factor; PKA, protein kinase A; PLP, pyridoxal 53 phosphate; RARβ, retinoic acid receptor beta; RD, restricted diet; rIL-2, recombinant interleukin-2; 54 RNS, reactive nitrogen species; ROS, reactive oxygen species; RSS, reactive sulfur species; SA-6MP, 55 S-allyl-6MP; SA-6MPR, S-allyl-6-MPR; SAH, S-adenosyl homocysteine; SAM, S-adenosylmethionine; 56 SAH, s.c.; subcutaneous; SCEs, sister chromatid exchanges; Smac, second mitochondria-derived 57 activator of caspase; SOD, superoxide dismutase; SP, side population; STAT, signal transducer and 58 activator of transcription; TAM, tamoxifen; TMZ, temozolomide; TNF, tumor necrosis factor; TNFR, 59 tumor necrosis factor receptor; TRADD, TNFR-associated death domain; SH2, Src-homology 2 60 domain; VEGFs, vascular endothelial growth factors.

GRAPHICAL ABSTRACT



66

1. Introduction

67 The prevalent perception that natural products are largely harmless conversely to their 68 chemically synthesized counterparts, although not entirely accurate, represents the most significant 69 reason for the growing consumer's preference for natural products and their rising attractiveness 70 in the pharmaceutical sector. In recent decades, the pharmaceutical industry has primarily focused 71 on high-throughput biochemical screening programs to discover and develop new drugs, but the 72 use of natural products as drugs has been around for a long time [1]. For instance, many foods or 73 spices have been largely exploited from ancient medicine to treat various disorders. Garlic, for 74 example, has been used from the times of ancient Egypt first and ancient Greece later to increase 75 the strength of the slaves and protect them from diseases. Garlic could also be considered a form 76 of "doping" since athletes of classical Olympic Games chewed garlic cloves to enhance their 77 performances.

78 Garlic contains several sulfenic acids that form thiosulfonates, of which allicin is the most 79 abundant and the one with the most potent active biological properties. Allicin has many health-80 promoting properties, such as cardioprotective, antimicrobic, cholesterol-lowering, anti-81 inflammatory, and antitumor. Allicin demonstrated an antitumor activity on a variety of tumor types 82 (leukemia [2,3], lymphoma [4], cholangiocarcinoma [5], gastric [6], hepatic [7], breast [7–9], lung 83 [7,10,3], prostatic [7], renal [11], colon [9,12–15,3], endometrial [9], cervical [16], and bladder 84 cancer [17]) (tables 1 and 2). Interestingly, allicin showed the ability to counteract all the phases of 85 carcinogenesis. This broad spectrum of action is due to its reactive nature and the ability to interact 86 with different molecular targets. Here, we present an overview of allicin's antitumor potential. 87 Some review articles about the anticancer potential of garlic have already been published [18,19]. 88 However, they mainly focused on garlic products, *i.e.*, extracts or other complex products, or several 89 bioactive components of garlic. As far as we know, there are no extensive and exhaustive reports

90 on the anticancer potential of the sole allicin, which represents the most characterized and active 91 compound with antitumor activity found in garlic. As a matter of fact, few reviews [20,21] describe 92 the anticancer and chemopreventive effect of allicin on gastric cancer, but as far as we know, no 93 report collects the knowledge of its anticancer properties on different tumor types. Thus, in this 94 review, we aim to broaden the range of data: we will describe and critically analyze allicin's 95 anticancer mechanisms straightforwardly and comprehensively by including all tumors for which an 96 effect of allicin has been demonstrated. To fulfill our aim, we decided to focus on the different in 97 vitro and in vivo cellular and molecular mechanisms through which allicin mediates its anticancer 98 effects: DNA damage protection, induction of cell death, inhibition of cell proliferation, and block of 99 angiogenesis and metastasis formation. Furthermore, to define the actual antineoplastic potential, 100 allicin's pharmacokinetics, bioavailability, and selectivity towards tumor cells are outlined.

101

2. Allicin chemistry and biosynthesis

Allicin [S-(2-propenyl)-2-propene-1-sulfinothioate] is a lipid-soluble sulfenic acid thioester Being very unstable, some processing and storage conditions such as concentration, pH, polarity of the medium and temperature can easily lead to a spontaneous decomposition to secondary organosulfur compounds as reported in figures 1, 2 and 3 [23–28].



107

108 Figure 1. Enzyme-catalyzed biosynthesis of allicin

Cysteine is transformed to alliin (S-allyl-L-cysteine sulfoxide) that is hydrolyzed by the alliinase enzyme producing
 dehydroalanine and allyl sulfenic acid where two molecules were combined to form allicin.

112 In the allicin biosynthesis (Figure 1), cysteine is converted to alliin (S-allyl-L-cysteine sulfoxide) 113 [1,24]. The alliinase enzyme hydrolyzes and splits alliin giving dehydroalanine and allyl sulfenic acid 114 that is highly reactive and unstable at room temperature, where two molecules come together to 115 form allicin [29,30]. Allicin represents about 70% of total thiosulfinates produced by mechanical 116 crushing of the garlic cloves [22,31], where alliin and alliinase are enclosed in different 117 compartments within garlic clove. When raw and fresh garlic is consumed, the acts of chopping, 118 crushing, chewing, or blending activate alliinase, causing maximum allicin production in less than 119 6s, well before reaching the intestinal tract [32,33]. Of note, the activity of alliinase is dramatically 120 affected by the gastrointestinal environment (gastric acid, intestinal proteases) and temperature, 121 being optimum at pH 7.0 and 35 °C and becoming inactivated at pH values below 3.5 or with heating 122 [34]. For this reason, in order to protect alliinase enzyme and magnify the efficiency of garlic 123 supplements, many brands of garlic supplements have adopted enteric-coated formulations to 124 prevent stomach disintegration [35].

Noteworthy, immediately after the discovery of alliin, it has been found that some bacteria,
 including some commonly present in the intestinal tract, possess alliinase activity [36–39]. It is also

possible that body organs could have alliinase activity, although none has yet been reported;
however, antibodies to alliinase have been detected in human serum [40].

Further studies are needed to better understand alliinase activity and consequently allicinconversion in humans.

131 **3.Bioavailability and Pharmacokinetics**

132 *In vitro* studies have shown that when allicin (or its transformation compounds) is added to 133 fresh blood, it is rapidly metabolized (half-life, <1 min) to allyl mercaptan with S-134 allylmercaptoglutathione (GSSA) as an intermediate [41]. It may have been formed from action of 135 glutathione (GSH) on components containing the C_3H_5 -S-moiety (Figure 2).

136



137

Figure 2. Metabolic fate of allicin. The Figure summarizes the proposed metabolic pathways of allicin. Glutathione
 (GSH); S-allylmercaptoglutathione (GSSA); glutathione disulfide (GSSG); S-adenosylmethionine (SAM); S-adenosyl
 homocysteine (SAH).

141 As an alternative, in the digestive tract, where both protein-bound cysteine and free cysteine-

142 released by protein hydrolysis-are present, allicin and its derived compounds can quickly react with

143 the amino acid cysteine to form S-allylmercaptocysteine (Figure 2) [41,42]. When S-144 allylmercaptocysteine is incubated in fresh human blood, (but the reaction occurs also in epithelial 145 cells) [41], it is metabolized to stable amounts of allyl mercaptan, even if efforts to find it in the 146 blood, urine, or stool after consuming garlic or allyl mercaptan have failed, indicating that it is rapidly 147 metabolized [43] (Figure 2). This is confirmed in humans, where the presence of allyl mercaptan is 148 transitory, reaching maximum levels in 1-2 min and disappearing by 1h, making it inappropriate as 149 an indicator of allicin bioavailability [44,45]. Under the acidic gastric conditions in the stomach, 150 allicin rapidly hydrolyzes to thioacrolein, which is metabolized by the enzyme methyltransferase 151 with S-adenosylmethionine (SAM) as methyl donor, to allyl methyl sulfide (AMS) (Figure 2). Both 152 thioacrolein and AMS are excreted in the urine and are unmistakable in the unpleasant "garlic 153 breath" [35,45-47].

154 To better clarify the fate of allicin in the human body, it became increasingly clear that the 155 metabolite to be followed was AMS. Human breath AMS has been found to reach maximum levels 156 in 4h and to persist for over 30h, proving that it is a product of allicin systemic metabolism [32,48]. 157 Furthermore, Lawson and Wang [32] conducted studies on human breath and came to important 158 conclusions: the area under the 32-h breath AMS concentration curve (AUC) is linearly proportional 159 to the amount of allicin consumed; AMS is the main breath metabolite of allicin, being allyl 160 mercaptan only a temporary intermediate in the formation of AMS from allicin (Figure 2); allicin-161 derived secondary products (Figure 3) are also mainly metabolized to AMS (Figure 2); AMS is an 162 active metabolite and responsible for the ability of allicin to increase breath acetone levels (Figure 163 2). Taucher et al. [48] showed that consumption of a single dose of crushed garlic doubled the breath 164 acetone output over a 32h period, an effect that may indicate an increase in triglyceride 165 metabolism. For this reason, acetone can also be used as possible marker for allicin bioavailability.



166

Figure 3. List and structure of some of the organosulfur compounds from allicin spontaneous decomposition and metabolism.
 Some more pharmacokinetic parameters derived from an animal study showing that ³⁵S labeled allicin is at least 79% absorbed within 30-60 min (Tmax) after oral administration. The mean
 total fecal and urinary excretion was 85.5% after 72h [49].

173 In a rat liver perfusion study, a remarkable first-pass effect of allicin was noted: 90% of allicin 174 concentration decreased just after 3 min while 99% disappeared after 6 min. The secondary 175 organosulfur compounds such as diallyl disulfide formed and later allyl mercaptan, ajoenes and 176 vinyl-dithines (Figure 3) were also observed in the collected bile as well as in the liver [50–52]. The 177 liver can metabolize diallyl disulfide back into allicin [53].

Overall, the studies here reviewed indicate that the consumption of allicin resulted in the same amount of breath AMS (and at similar rates) as consuming equivalent amounts of smaller molecules (AMS and allyl mercaptan). It can be concluded that allicin absorption in humans is at least 95% [32]. Obviously, the allicin bioavailability is highly influenced by its formulation. Comparing types of garlic products, allicin bioavailability registered in healthy subjects 32h postconsumption was 36–104% for enteric tablets, 26–109% for garlic powder capsules, 80–111% for
non-enteric tablets, 30% for roasted, 16% for boiled, 66% for acid-minced, and 19% for pickled garlic
foods [35].

More specific and extensive studies on metabolism and excretion refer to garlic. Since allicin in turn converts into other organosulfur compounds naturally occurring in garlic (Figure 3), it can be assumed and extrapolated that the metabolic behavior and fate of allicin will have commonalities and overlaps with those of garlic. Reduction, methylation, oxidation, GSH conjugation represent the main metabolic reactions to which allicin and its derived compounds are subjected. Because of the disparate *in vitro* systems, *in vivo* models and human ethnic groups employed in these studies, the metabolic pathways could be different for each individual organosulfur compound [52].

Data from clinical studies give some more in-depth information regarding excretion. In fact, by GC-MS analysis, it was shown that allyl mercaptan, AMS, allyl methyl disulfide, diallyl sulfide, diallyl disulfide, and diallyl sulfone were the components, with AMS being the most abundance detected in the human breath soon after the ingestion of raw garlic and commercial garlic products [52], whereas AMS, allyl methyl sulfoxide and allyl methyl sulfone were the main metabolites found in human milk and urine [46,47,54].

Again, further efforts are needed to definitely clarify the pharmacokinetics of allicin, process complicated by the high reactivity of the molecule and by its intense and quick metabolism. Based on human studies, we can conclude that the metabolism of allicin generated various secondary products, AMS being the most representative metabolite. Extrapolating data from garlic we can assume similar elimination routes [32].

Although allicin is short-lived and poorly stable, it can easily cross cell membranes due to its hydrophobic nature, indicated by its calculated logP of 1.35. Accordingly, it is readily membrane permeable and taken up by cells [24,29]. Allicin has been shown to create transient pores as it

crosses membranes as if no membrane were present (at the rate of diffusion). Its diffusion through the lipid bilayer does not cause membrane leakage, fusion or aggregation. Temporary pore formation leads to a transient depolarization, *i.e.*, a decrease in the membrane potential (ΔE_m). Membrane permeabilization appears to be a physical effect and is unrelated to any chemical reactivity of allicin [55]. These findings raise the possibility that in biological systems allicin can pass very rapidly through cell compartments, where it rapidly reacts with free thiol groups and exerts its biological and pharmacological effects [56].

214

3.1 Improvement of allicin bioavailability

Allicin goes through a fast metabolism through which it is quickly inactivated. This behavior clearly limits the clinical potential of this compound. Moreover, allicin is characterized by chemical instability and short life. For example, it can be inactivated by heating or at a pH below 3.5 [57]. Therefore, enteric-coated formulations have been developed to hamper stomach disintegration of many commercial garlic supplements and protect against allinase enzymes [35]. A microparticulate formulation, in which alliinase and alliin are individually encapsulated inside microspheres, has been developed for pulmonary administration [58].

222 However, a fascinating and so far convincing strategy to overcome those limitations is the in-223 situ generation of allicin using tumor-specific delivery systems. For example, Chhabria et al. [59] 224 detected a particular tumor antigen (CA19-9) expressed mainly by pancreatic cancer cells and 225 created a specific antibody conjugated with alliinase. The treatment of pancreas ductal 226 adenocarcinoma cells (MIA PaCa-2) with the alliinase-conjugated antibody followed by treatment 227 with free alliin provoked the *in-situ* synthesis of allicin and consequent selective cell death. 228 Accordingly, cytotoxicity has been recorded only in pancreatic tumor cells. Indeed, the same 229 treatment has been directed on different CA19-9-negative cell lines, such as liver (HepG2), ovary

(OAW42), prostate (PC3), breast (MCF7) cancer cells, and human dermal fibroblasts (HDF) and no
cytotoxicity has been recorded [59]. On MIA PaCa-2, the treatment with this conjugate system,
followed by 50 μM alliin, promoted proliferation inhibition and apoptosis. In particular, it promoted
the acetylation of the histone H3, to which the authors ascribe the ability to activate p21^{Waf1/Cip1} and
the following cell-cycle arrest in the G1 phase. Apoptosis was associated with oxidative stress, as
indicated by the increase in ROS levels, depletion of GSH, and activation of caspases cascade [59].

236 A similar strategy has been applied to target human ovarian cancer cells. Instead of targeting 237 a tumor antigen, the natural compound daidzein has been used to bring alliinase to the tumor cells. 238 This compound is known to bind lipids on cell surfaces and facilitate endocytosis in a tumor-239 dependent way. Accordingly, the system daidzein + alliinase (300 nM) together with alliin (1 mg/mL) 240 was cytotoxic only on ovarian cancer cell lines (ES-2, MLS, OVCAR3), while no effect has been 241 recording on pancreatic (Panc-1, p34 and COLO 357) cancer cell lines. In this case, the mechanism 242 of action has not been investigated, but the selectivity and antitumor effect of the system has been 243 confirmed in vivo. ES-2-Luc-bearing mice injected with the daidzein conjugate and alliin showed 244 significant inhibition of tumor growth proliferation without any toxic or side effect. Localization 245 studies showed a tropism of the system toward the tumor, which was found 5 times more in 246 malignant tissues than in normal ones. However, the most interesting outcome of this study is that 247 daidzein + alliinase (150 µg) followed after 1.5 h by alliin (3 mg) every day, 5 days per week and 248 repeated for additional 5 days after a 2-day break, inhibited tumor growth similar to cisplatin (6 249 mg/kg/week), but without inducing the same toxic events [60]. A similar specific antitumor effect 250 has been recorded on N87-xenografted athymic nude mice treated with an alliinase conjugate 251 directed to the tumor marker ErbB2 and treated with alliin to activate the local reaction and 252 synthesize allicin in situ [61].

253 Even liquid tumors can be targeted by an alliinase conjugate; it is sufficient to find suitable 254 markers. Arditti et al. [62] acknowledged CD20 antigen as the specific marker to target B-CLL and 255 lymphoma; thus, they build an alliinase conjugate exploiting the CD20-binding specificity of 256 rituximab. As for the previous studies, the alliinase-rituximab conjugate has been administered 257 before allicin to generate allicin *in situ*. In this study, in addition to lymphoma cell lines (MCL and 258 EBV) and an *in vivo* model, the system has been tested on 5 patients' samples of B-CLL peripheral 259 blood mononuclear cells (PBMC). In all three settings (in vitro, in vivo and ex vivo), the rituximab-260 alliinase + alliin system demonstrated a clear antitumor activity. On the two lymphoma cell lines and 261 five ex vivo samples, it promoted cell death while no effect was recorded on CD20 negative cells, 262 such as neuroblastoma LAN-1 and normal T cells. In vivo, a single injection of the conjugate (80 263 µg/mouse) followed by repeated administration of alliin (3 mg/mouse, twice a day, during 3 days) 264 induced a significant reduction of the amount of B-CLL cells [62]. This outcome is fascinating since 265 it seems to be a valid alternative to free allicin, which, as will be discussed in paragraph 6, causes 266 hemolysis and eryptosis due to the lack of selectivity towards tumor cells [63].

267

4. Antitumor effects of allicin

268 **4.1** Inhibition of DNA damage and anti-inflammatory effects

269 Since DNA damage is a prerequisite for initiating the entire carcinogenic process, it is critical 270 to identify natural compounds that can prevent or inhibit this type of harm.

Allicin can promote: 1) indirect DNA protection (antioxidant activity and modulation of oxidizing enzymes), 2) direct DNA protection 3) immunomodulation.

273 Reactive oxygen species (ROS) and reactive nitrogen species (RNS), known as free radicals, are 274 very reactive molecules. ROS and RNS are commonly produced by cellular metabolism as they 275 participate in several signaling processes. However, when the amount of ROS and RNS overwhelms

the capacity of antioxidant systems, oxidative and nitrosative stress occurs. Then, ROS and RNS damage cellular components such as DNA and compromise cellular functionality. For this reason, free radicals play a role in the development of cancer, and quenching them represents a strategy to prevent tumor initiation [64].

Several studies have shown allicin to have antioxidant activity in different ways. Indeed, allicin scavenges oxygen radicals directly [65], suppresses the activity of oxidizing enzymes by direct interaction through thiol-disulfide exchange reactions [66,67] or inhibits the expression of mRNA encoding such enzymes [68]. Allicin has also exhibited antioxidant effects through increasing gene expression of genes encoding phase II detoxifying enzymes [69].

Allicin, at the concentrations 1.25 mg/mL (7.70 mM), demonstrated antigenotoxic activity in a mutant bacterial cell assay in which it appeared effective in decreasing methotrexate genotoxicity, which is in part mediated by free radicals [70].

288 Furthermore, due to its antioxidant activity, allicin exerted antigenotoxic action against 289 estradiol-17-β-induced genetic damage in normal human lymphocytes under metabolic activation 290 [71]. Metabolic activation of estradiol-17-β produces secondary metabolites, which are rapidly 291 oxidized to o-quinones. The o-quinones generate free radicals, which are the actual effectors of 292 estrogen genotoxicity [72,73]. Structural chromosomal aberrations (CAs) and sister chromatid 293 exchanges (SCEs) were employed as cytogenetic endpoints to quantify DNA damage. Lymphocytes treated with increasing concentrations of estradiol 17- β (10-20-40 μ M) yielded a concentration-294 295 dependent amount of CAs and SCEs. In contrast, the treatment with estradiol-17-β plus allicin (5, 296 10, and 15 µM) significantly reduced the incidence of both CAs and SCEs in a concentration-297 dependent manner, demonstrating that allicin counteracts the genotoxic potential of estradiol 17β 298 [71].

299 However, in another co-treatment setting, allicin (5 - 100 µM) failed to protect cellular DNA 300 from H₂O₂ damage. Probably, the entity of the oxidative stress was too great to be counteracted. 301 Still, in the same condition, allicin protected DNA from the damage induced by the DNA alkylating 302 agent methyl methanesulfonate (MMS) in a concentration-dependent manner. In this case, it is 303 presumed that allicin acted as a nucleophilic agent able to intercept and bind MMS before it could 304 electrophilically attack DNA [74]. In the same study but a different setting, allicin was also tested for 305 its ability to directly interact with not-oxidizing mutagenic compounds and decrease their 306 mutagenicity. Pre-treatment of human hepatoma HepG2 cells with increasing concentrations of 307 allicin (range 5 - 100 μ M) prevented the genotoxic effect of aflatoxin B1 (AFB1) starting at 5 μ M 308 concentration, but could not prevent the toxicity of other agents such as benzo(a)pyrene and N-309 nitrosodimethylamine. Likely, this effect was due to the modulation by allicin of phase I and II 310 enzymes specifically involved in AFB1 metabolism [74].

311 Inflammation plays a key role in enhancing carcinogenesis. Inflammation is a defense 312 mechanism that is triggered by physical or chemical tissue damage. During the inflammatory 313 process, immune cells are attracted to the site of injury, where they produce large amounts of 314 cytokines and chemokines and eliminate the damaging agent by stimulating the abundant 315 production of ROS and RNS [75]. Relevant proinflammatory cytokines are tumor necrosis factor 316 (TNF)- α and interleukin 1 β (IL-1 β), which can stimulate ROS production and the expression of the 317 enzyme inducible nitric oxide synthase (iNOS) in different cell types [75]. When the tissue damage 318 occurs over a short period, these reactive species are less likely to damage the DNA of cells around 319 the inflammation area, but when it develops a chronic course, the risk of DNA damage and 320 carcinogenesis induction increases. TNF- α is a proinflammatory cytokine mainly secreted by 321 macrophages, the predominant immune cell population during chronic inflammation [75]. The 322 synthesis of TNF- α , as well as that of other critical proinflammatory cytokines, such as IL-1 β , and

323 chemokines, such as IL-8, is mediated by nuclear factor kappa-light-chain-enhancer of activated B 324 cells (NF- κ B) [76]. However, TNF- α itself is a primary inducer for NF- κ B activation [77], suggesting 325 the existence of a mutual positive feedback regulation [78]. NF-κB is a heterodimer that operates as 326 a transcription factor. It is located in the cytoplasm in an inactive form complexed to the NF-KB 327 inhibitory protein IkB. In response to a wide range of stimuli, the activation of the enzyme IkB kinase 328 (IKK) can occur, resulting in phosphorylation of IkB, which is ubiquitinated and then degraded, 329 thereby releasing NF- κ B [76]. NF- κ B migrates from the cytoplasm into the nucleus and operates as 330 a transcription factor, inducing the expression of both antiapoptotic and proinflammatory genes, 331 including TNF- α and iNOS. In a study performed on two rectal adenocarcinoma cell lines, HT-29 and 332 Caco-2, the ability of allicin to modulate both spontaneous and TNF- α -induced proinflammatory 333 cytokine secretion was assessed [79]. The results show that, in both cell lines, allicin inhibited 334 spontaneous and TNF- α -induced secretion of IL-1 β starting from a concentration of 20 mM in a 335 concentration-dependent manner. Similarly, spontaneous (50% reduction at 20 mM) and TNF-α-336 induced (50% reduction at 40 mM) secretion of IL-8 was also inhibited in both cell lines [79]. Thus, 337 the inhibition of the secretion of these cytokines and chemokines by allicin appears to depend in 338 part on the inhibition of IL-1β and IL-8 mRNA levels, as well as by blocking NF-κB release through 339 the inhibition of IkB degradation [79]. Thus, according to these results, allicin would suppress the 340 release of proinflammatory signals that may be involved in cancer genesis by targeting the 341 expression and secretion of cytokines and chemokines. The immunomodulatory effects of allicin 342 were also observed in vivo in a mouse model of immune-mediated hepatitis. Allicin (21mg/kg/day 343 for 7 days) was able to inhibit TNF- α secretion, NF- κ B activation, and iNOS expression [80].

344 **4.2 Induction of cell death**

345 The large majority of the studies regarding the antitumor potential of allicin showed apoptosis 346 as the primary cell death mechanism, followed by parthanatos and autophagy (Figure 4).

347 For a long time, apoptosis has been considered the only programmed form of cell death 348 instead of the not—programmed, accidental, and passive necrosis. In 1973, three types of cell death 349 were identified: i) type I or apoptosis; ii) type II or autophagy; iii) type III or necrosis. So far, it is clear 350 that programmed cell death is not a unique and single process, but in different situations, in 351 different circumstances, and depending on a plethora of not yet fully understood factors, cells can 352 commit suicide in different ways. Currently, at least 12 types of regulated cell death modalities have 353 been discovered and characterized. Some of them run through partially overlapping molecular 354 mechanisms, which let us presume that they represent backup plans when one way is not 355 functioning. For instance, apoptosis has for a long time been confused with parthanatos, which was 356 called caspase-independent apoptosis, given the similar mediators and morphological 357 characteristics of these two types of cell death [81].

358 Apoptosis occurs through two distinct pathways: the receptorial or extrinsic pathway and the 359 mitochondrial or intrinsic pathway. These two cascades have common pivot points and converge in 360 activating the same effectors: caspases 3, 6, and 7. Extrinsic apoptosis is triggered by extracellular 361 signals which activate the so-called death receptors, such as FAS or TNF receptor (FASR; TNFR). The 362 receptor-ligand binding induces an alteration that promotes the recruitment of cytoplasmatic 363 adaptor proteins such as FAS-associated death domain (FADD) and TNFR-associated death domain 364 (TRADD) and allows the formation of a complex called DISC (death-inducing signaling complex). This 365 receptor-adaptor complex binds the initiator procaspase 8. The latter protease is proteolytically 366 activated in caspase 8, which activates other proteins, including the effector caspase 3 or BH3 367 interacting domain death agonist (BID) protein. Caspase 3, then, induces the typical apoptotic 368 morphological and cellular changes, which brings to the degradation of the cell, while BID represents

the converging point with the intrinsic pathway. Subsequently, BID translocates to the mitochondrion, promoting the activation of Bcl-2-like protein 4 (Bax) and Bcl-2 antagonist/killer (Bak) [82].

372 In addition to caspase 8, the intrinsic pathway is activated by intracellular insults due, for 373 instance, to oxidative stress or DNA damage. In this case, mitochondrial proteins belonging to the 374 Bcl-2 family, such as cytochrome-c and Smac/DIABLO (second mitochondria-derived activator of 375 caspase/direct inhibitor of apoptosis-binding protein with low PI), are recruited and activated. For 376 the apoptotic program to begin, BID must bind Bcl-2 to release Bak and Bax. The two free molecules 377 oligomerize and interact with the mitochondrial membrane forming pores from which 378 mitochondrial factors such as cytochrome-c, Diablo, and Ca²⁺ ions emerge. In the cytoplasm, 379 cytochrome-c binds apoptotic protease activating factor-1 (Apaf-1) and ATP. This complex then 380 binds procaspase 9, forming the apoptosome. Simultaneously, Diablo, which has a binding domain 381 that mimics the cut procaspase small unit, binds apoptosis inhibitor proteins (IAPs), inhibiting them. 382 Caspase 9 then activates the effector caspases -3, -6, and -7 inducing the cellular effects typical of 383 apoptosis, such as chromatin condensation and DNA fragmentation [82]. However, the same 384 morphological effect can be obtained without the involvement of caspases. In this case, the main 385 character is mitochondrial apoptosis-inducing factor (AIF). AIF is poured from the mitochondria in 386 the cytoplasm and then into the nucleus, inducing parthanatos. Parthanatos has been first described 387 as a caspase-independent apoptosis, but the characterization of the specific molecular mechanism 388 made a distinction with apoptosis necessary. Parthanatos shares with apoptosis the same players, 389 such as PARP (Poly (ADP-ribose) polymerase)-1, which is responsible for the nuclear translocation 390 of AIF. Also, AIF activates JNK (c-Jun N-terminal kinase) and upregulates the expression of Bcl-2, Bax, 391 and Bad, which are essential molecules in the regulation of cell death [81].

392 On the gastric cancer cells SGC-7901 and glioma U251, allicin 30 µg/mL (184.4 µM; 48h) 393 triggered both intrinsic and extrinsic apoptotic pathways (table 1). On U251, it increased protein 394 and mRNA levels of FAS ligand (FASL), Bax and caspase 3 and decreased Bcl-2 [83], while on SGC-395 7901 it promoted the disruption of mitochondrial potential and increased the protein and RNA 396 expression of cytochrome c, Bax and FAS, caspase 3, 8, and 9 expression and activity [84] (table 1). In the same cell line, another study showed that a 10-time lower concentration of allicin also 397 398 triggered apoptosis. However, in the latter study, it is not clear how apoptosis was detected, and no 399 molecular mechanism was investigated [85] (table 1). Starting at lower concentrations, the intrinsic 400 pathway has been activated also on oral tongue squamous cell carcinoma Tca-8113 and SCC-25 [12] 401 μ g/ml (73.9 μ M); 48h] [86], luminal A (MCF7; 40 μ M; 24h) and triple-negative (HCC-70; 20 μ M; 24h) 402 breast carcinomas [87], murine fibrosarcoma L929, SW480 (50 μM, 24h) [88] or Siha (50 nM; 72h) 403 [16], gastric cancer HGC27 and AGS [10 μg/mL (61,61 μM); 48h] [89], colorectal cancer Caco-2, HT29 404 (500-1000 μM) [2,90,91], and HCT116 (25 μM; 48h) [13,14], lymphoma EL-4 (4 μg/mL (24.6 μM); 405 24h) [13,92] and L5178Y (1,09 mM; treatment time not specified) [4], leukemia HL-60 (5 μM; 16h) 406 and U937 (20 µM; 16h) (table 1)[2].

407 In gastric AGS tumor cells, allicin showed a different behavior depending on the concentration. 408 At 10 μg/mL (61.61 μM; 48h), allicin induced apoptosis [89], while in another study, allicin 20 μg/mL 409 (123,22 µM; 24h) showed an interesting although puzzling mechanism of action (table 1) [93]. The 410 apoptotic potential of allicin 10 µg/mL has been attributed to the ability to modulate a specific 411 microRNA (miRNA). miRNAs are a group of small (18-22 nucleotides) non-coding single-stranded 412 (ss) RNA sequences that have been identified in many organisms. miRNAs act by negatively 413 regulating gene expression at the post-transcriptional gene silencing level. They recognize specific 414 mRNA targets and determine their degradation or translational repression. Some miRNAs, such as miR-383-5p, have been shown to play a role in cell proliferation, apoptosis, and differentiation. In 415

416 particular, miR-383-5p is a specific tumor suppressor in gastric cancer, and its inhibition is related 417 to poor prognosis. Nonetheless, it is strictly connected with cell proliferation and metastasis. On 418 HGC27 and AGS gastric cancer cells, allicin 10 μ g/mL (61.61 μ M, 48h) promoted intrinsic apoptosis 419 by upregulating miR-383-5p and its downstream effector ERBB4 and impairing phosphoinositide-3-420 kinase/protein kinase B (PI3K/Akt) signaling cascade (table 1) [89] . However, as foretold, a puzzling 421 mechanism arose when allicin concentration was doubled. Indeed, doubling the concentration of 422 allicin did not affect either caspase activity nor apoptosis. In particular, the study's dataset [93] 423 showed the ability of allicin to promote cell death without the activation of caspase 3 and PARP but 424 mediated by protein kinase A (PKA), which triggered Bax, AIF, and, ultimately, cell death. These data 425 are odd since PKA is usually linked to apoptosis, but no caspases activation has been recorded. 426 Furthermore, AIF suggests the involvement of parthanatos, but no PARP activation has been shown 427 [93]. The only consistent reference we found which links PKA and AIF concerns cisplatin-resistant 428 HepG2 cells treated with cathepsin B. In this study, cathepsin B activated a cascade that foresaw the 429 activation of the PKA/PP2 A/IKK axis and the consequent initiation of AIF-scramblase mediated "eat 430 me" signal release and cell death. But still, in this axis, the involvement of caspases is hypothesized, 431 and authors refer to this cell death as apoptosis [94]. Thus, this mechanism cannot be applied to 432 allicin-mediated AGS cell death, and only further studies can better characterize this odd mechanism 433 of action.

Although many studies showed the ability of allicin to induce apoptosis, few of them unraveled the precise molecular mechanisms of action. A couple of papers showed a direct effect on GSH or on the oxidative cellular *status* in general, while others the involvement of p53, the activation of mitogen-activated protein kinases (MAPKs), or signal transducer and activator of transcription (STAT) 3 (Figure 4).





Figure 4. Schematic representation of allicin-mediated cellular death pathways. GSH (glutathione); GSSG (glutathione
disulfide); ERK (extracellular signal-regulated kinase); JNK (c-Jun N-terminal kinase); STAT3 (signal transducer and
activator of transcription 3); Bax (Bcl-2-like protein 4); Bcl-2 (B-cell lymphoma 2); APAF-1 (apoptotic protease activating
factor-1); AIF (apoptosis inducing factor); PARP-1 (Poly (ADP-ribose) polymerase)-1); PAR (poly(ADP-ribose). Created
with BioRender.com.

4.2.1 Oxidative cellular status and GSH

446	Although allicin is a reactive sulfur compound that quickly oxidizes -SH groups, such as those
447	in GSH or those present in any cysteine residue of different proteins, it also acts as an antioxidant
448	thanks to a sort of rebound effect. Indeed, oxidative stress can be detrimental for cells, but it also
449	activates an antioxidant response mediated by oxidative stress protection responses [1].
450	Certainly, as a consequence of oxidative stress in general, and GSH drop in particular, as a
451	defense mechanism, cells promote the activation of nuclear factor-erythroid factor 2-related factor
452	2 (Nrf2) and its translocation into the nucleus. Nrf-2 is a critical transcription factor that modulates
453	more than 1000 genes in normal and stressed circumstances. Nrf2 endorses the synthesis of several
454	proteins having different roles, such as an antioxidant, detoxifying, anti-inflammatory [95]. It is not
455	known which effectors are involved in allicin-mediated cytotoxicity, but in HCT116 colon cancer

456 cells, it is clear that Nrf2 is essential to promote cell death. Indeed, allicin 10 μ g/mL (61.61 μ M) 457 promoted Nrf2 translocation into the nucleus and its silencing inhibited the anticancer effect of 458 allicin (table 1) [14].

459 Despite the antioxidant properties of garlic and allicin itself, which can be ascribed to the 460 activation of Nrf2 and the induction of phase II detoxification enzymes, as already anticipated, the 461 chemical structure of allicin qualifies itself as a reactive sulfur species (RSS). Indeed, under 462 physiological conditions, allicin effortlessly penetrates cellular membranes thanks to the 463 thiosulfinate moiety. There, it reacts with thiol groups and oxidizes biomolecules, such as GSH 464 cysteine residues. For instance, in leukemia cells (HL-60 and U937), the reaction allicin-GSH 465 represents the trigger to ignite apoptosis. Precisely, allicin 5 µM rapidly moved inside the cells and 466 oxidized GSH in glutathione disulfide (GSSG), unbalancing the GSH/GSSG ratio with a subsequent 467 decreased cellular reduction potential, followed by mitochondrial damage that started the intrinsic 468 apoptotic pathway. Subsequently, cytochrome c release and activation of caspases 3 and 9 469 concluded the process [2]. Moreover, GSH depletion and oxidative stress are crucial for allicin to 470 promote apoptosis in glioblastoma cells, where pre-treatment with the GSH precursor N-471 acetylcysteine (NAC) significantly reduced the proapoptotic activity of allicin 90 µM [96]. Likewise, 472 very high concentrations of allicin (500 µM) promoted GSH depletion on colon carcinoma Caco-2 473 cells after 3 and 6 h but not on HT-29 cells [90]. On HT-29 cells, GSH was depleted at much lower 474 allicin concentrations (37.5-70 µM), but longer treatment (24h) [91]. In the same concentration 475 range, allicin (32 µM) promoted a drop of GSH levels on MCF7 cells starting after 10 minutes, 476 followed by a recovery to basal levels after 3 hours. In this case, probably, the GSH drop was caused 477 by a direct conjugation allicin-GSH and only to a lesser extent by oxidization of GSH in GSSG, as an 478 elevation of these molecule levels was not detected. In addition, the authors of the study did not 479 investigate the mechanism underlying the fast GSH recovery, but they proposed that it may be due

480 to the stimulation of its synthesis *de novo* as a compensatory response of the cell to the quick GSH481 drop [9].

482 **4.2.2. p53**

483 In hepatic cancer cells, the activity of p53 was crucial for the cytotoxic activity of allicin. p53 is 484 commonly renown as the "guardian of the genome" and controls and pivots different death 485 processes, such as apoptosis [97], autophagy [98], and necrosis [99]. In the case of allicin and hepatic 486 cancer cells, p53 drove cells to go through autophagy, while its absence allowed apoptosis to take 487 over. Indeed, Hep3B characterized by a mutated- not functional-p53, and p53⁻-HepG2 responded to 488 allicin 35 µM (24h) with ROS-mediated apoptosis (increased Bax, caspase 3, 8, 9 levels and 489 decreased mitochondrial potential and Bcl-2 levels) and parthanatos (AIF, high-temperature 490 requirement A2 (HtrA2) and endo G increased levels, two additional markers of parthanatos). While, 491 at the same conditions, HepG2, characterized by a functional p53, went through autophagy (table 492 1) [100]. p53 probably plays a role in the allicin antitumor activity on subdermal glioblastoma 493 multiforme (GBM) infected with human cytomegalovirus (HCMV). Allicin 60 µg/mL (369.7 µM) 494 induced cell death by increasing p53 levels, reduced the inflammatory status promoted by the 495 HCMV infection on U87MG cells by counteracting the interferon β (IFN- β) and IL6 overexpression, 496 and directly fought virus infection [101].

497 **4.2.3 MAPKs**

The stress-activated protein kinases, or MAPKs family, represent one of the fundamental intracellular signal transduction systems in the monitoring and surveillance of cell survival, proliferation, differentiation, and cell death [102–105]. MAPKs include three main signaling branches, mediated by p38-MAPK, extracellular signal-regulated kinases (ERK), and JNK. In different

502 tumor models and at different conditions, allicin modulated all these three pathways. At 90 µM, 503 allicin promoted a caspase-independent regulated cell death and the phosphorylation of both p38 504 and ERK. In particular, the latter kinase was concretely linked to the ability of allicin to promote cell 505 death since its inhibition significantly decreased the cytotoxic potential of allicin [96]. p38 levels 506 were also increased by allicin in MGC-803 gastric carcinoma (6.16 µM) [106] and SK-N-SH 507 neuroblastoma cells (5 µM) [107], and in both cases, this event was supposed to be linked to the 508 ability to promote apoptosis [106,107]. Especially on SK-N-SH, the blockage of the p38-MAPK 509 cascade prevented the release of cytochrome c from the mitochondria and the subsequent cell 510 death [107]. In Jurkat leukemia T cells, allicin 10 µg/mL (61.6 µM) promoted cell death and the 511 activation of isoforms 1 and 2 of ERK through the oxidization of p21^{ras}, one of the most critical 512 upstream regulators of ERK (table 1) [3].

513 Finally, also the JNK branch has been found to be involved in the proapoptotic activity of allicin 514 in human SKOV3 ovarian cancer cells. At 25 μ g/mL (154.04 μ M, 24h), allicin promoted its 515 phosphorylation, which provoked the release of apoptosis mediators, such as Bax translocation and 516 cytochrome c release (table 1) [108].

517 Hypoxia is a common feature of solid and liquid tumors and a crucial marker of therapeutic 518 resistance. On A549 lung adenocarcinoma cells, allicin 40 µg/mL (246.34 µM, 72h) promoted 519 apoptosis and autophagy in normoxic and hypoxic conditions. In both situations, allicin induced 520 ROS-mediated apoptosis and autophagy. At a molecular level, allicin promoted a decrease in p38-521 MAPK and phosphorylated p38-MAPK, together with increasing the pJNK/JNK ratio. Although a 522 mechanistic study has not been performed, both the proapoptotic and autophagic potential of 523 allicin may be ascribable to the modulation of these mediators. In particular, it is known that p38-524 MAPK inhibition may lead to oxidative stress, which in turn activates the JNK pathway. In addition, 525 in hypoxic conditions, the negative modulation of p38-MAPK lessens the expression of hypoxia526 inducible factor (HIF) 1α and HIF 2α that is what allicin did in the study [109]. This event is interesting 527 because it denotes a strategy to enhance cytotoxicity in tumor cells in hypoxic conditions.

4.2.4 STAT3

529 Transcription factors belonging to the STAT family are key pawns in mediating the signaling 530 and functions of cytokines, interferons, and growth factors. STAT3, in particular, is an oncogene. It 531 is constitutively activated in many primary tumors and cancer lines, where its inactivation leads to 532 the arrest of cancer cell proliferation and cell death by apoptosis [110]. In HuCCT-1 533 cholangiocarcinoma cells, allicin 20 µM (24h) inhibited STAT3 activation and nuclear translocation 534 via Src-homology 2 domain (SH2)-containing SHP-1 overexpression. Specifically, allicin triggered the 535 caspase cascade activating caspases 9 and 3 and increased the Bax/Bcl-2 ratio (table 1). 536 Furthermore, overexpression of STAT3 reduced the antiproliferative effect, together with a reduced 537 modulation of the above-mentioned apoptotic markers [5], leading us to presume a link between 538 allicin apoptotic potential and STAT3.

539 **4.3 Inhibition of cell proliferation**

Allicin has been found to arrest the cell cycle in different phases, depending on the cell line and concentration used. Likewise, the mechanism of action is not unique, but arises from the modulation of different targets, such as telomerases, microtubules, ornithine decarboxylase 1 (ODC1) and others (Figure 5).



544

545 Figure 5. Schematic representation of allicin-mediated antiproliferative pathways. Created with BioRender.com. 546 547 At both low [leukemia HL-60 cells (5 μM, 20h)] [2] and high concentrations [SGC-7901 (616,14 548 μM, 24h [6], Caco-2 (500 μM, 24h) [90], and A549 (246.34 μM, 48h] [109]], allicin promoted a cell-549 cycle arrest at G2/M phase (table 1). In the case of SGC-7901, the antiproliferative effect could be 550 due to the inhibition of telomerase activity produced by allicin at the same conditions [6]. Telomeres 551 are non-coding, tandemly repeated nucleotide sequences found at the end of eukaryotic 552 chromosomes. They stabilize DNA, preventing chromosomes from wrapping around themselves or 553 recombining at their ends. Under physiological conditions, somatic cells present a very low or absent 554 telomerase activity, which does not allow the synthesis of new telomeres. Consequently, at each 555 replication cycle, telomere sequences shorten to a critical length, beyond which mitosis stops and 556 cells enter a phase known as "senescence". Senescent cells remain metabolically active, but their 557 gene expression is altered and division cycles slow down [111]. However, senescence, despite being 558 a physiological process, represents a way for tumor cells to acquire resistance towards 559 chemotherapeutics and can cause relapses and favor formation of metastasis [112]. For example, 560 double-negative MCF7 and triple-negative HHC-70 breast carcinoma cells develop resistance to doxorubicin (DOX) by going through senescence. Allicin 45 μ M (MCF7) and 20 μ M (HHC-70) showed a senolytic and antiproliferative effect on both these DOX-resistant senescent cells [87]. Thus, we can speculate that although allicin inhibits telomerase activity, it does not activate senescence, which for tumor cells represents a chemoresistant marker, but only blocks cell proliferation ending in cell death.

566 On non-resistant MCF7, instead of the senolytic effect, allicin promoted the accumulation of 567 cells in the G1 (20 μ M) or the G1 and G2/M phases (30 μ M) of the cell cycle, depending on the 568 concentration of the agent [9], but no mechanism of action has been investigated (table 1).

569 On A549 cells, the allicin-induced G2/M cell-cycle arrest, which was observed together with a 570 slight accumulation in the S and subG1 phases, is caused by ROS accumulation [109]. Allicin-induced 571 oxidative stress probably caused DNA double-strand breaks, which activated p53. p53, on its side, 572 promoted an increase in p21 and a decrease in cyclin B with the overall effect of the accumulation 573 of cells in the G2/M phase. Indeed, p21 is a potent inhibitor of cyclin-dependent kinases. The p21 574 protein binds to and inhibits the activity of cyclin-dependent kinase 1 (cdc2), which is essential for 575 entry into mitosis. The oncosuppressor p53 tightly controls the expression of p21, and it is through 576 this interaction that p53 can lead to cell-cycle arrest in the G2/M phase in response to stressful 577 stimuli of various nature [113].

578 Allicin 50 µM promoted the inhibition of cell proliferation on DS19 mouse erythroleukemia 579 cells and increased the acetylation rate of histone H4 [114]. Since acetylation of histone H4 plays a 580 critical role in loosening chromatin structures during DNA replication [115], the authors of the study 581 suggest that the two events can be correlated [114].

582 ODC1 is another indirect modulator of cell proliferation. It is a rate-limiting enzyme for the 583 biosynthesis of the polyamines putrescine, spermidine, spermine, which act as oncometabolite. 584 High levels of these proteins and ODC1 are often recognized in tumors such as neuroblastoma and

585 are directly involved in uncontrolled cell proliferation. Allicin 25 µM was able to block the 586 proliferation of different neuroblastoma cell lines (SK-N-AS, SK-N-Be(2)-C, and Kelly), together with 587 the suppression of ODC activity. In the same conditions, allicin still inhibited cell proliferation on SK-588 N-FI neuroblastoma cells, but no effect on ODC activity has been recorded, showing that ODC1 is 589 not the only allicin target that produces a cytostatic effect [116]. Accordingly, it has been 590 demonstrated that allicin directly interacts with microtubules resulting in the block of cell 591 proliferation, division, polarization, and migration. For instance, the exposure to the same 592 concentration of allicin (25 µM) resulted in inhibition of actin polymerization in human T-cells (1-h 593 treatment) [117] and modification of actin cytoskeleton on L929 (10 min treatment) [118].

594 On NIH-3T3 mouse fibroblasts, allicin 0.5 μ M provoked microtubule depolymerization within 595 30 min, inducing a sudden cytostatic effect without causing cytotoxicity. In particular, allicin 596 inhibited cell division by disrupting spindle formation during mitosis. As for the impact on GSH, the 597 direct interaction and antagonization of microtubules polymerization are due to the reaction with 598 the -SH groups of allicin through a thiol-disulfide exchange reaction with tubulin [119]. It is 599 interesting to highlight that the concentration needed to antagonize microtubules polarization and 600 cell proliferation is notably lower than the average concentration of allicin necessary to induce 601 cytotoxicity on different tumors cells, thus probably linked with a lower probability of toxic events. 602 However, it has also been seen that this effect on microtubules is slowly reversible. Although 603 reversibility could represent a throwback in allicin antitumor potential, an escamotage can be used 604 to overcome it and exploit the use of low concentrations of allicin. For instance, low concentrations 605 of allicin sensitize cancer cells to cytotoxic compounds. In other words, cells could be treated with 606 allicin and, after few minutes, with a cytotoxic compound. In this way, the concomitant use of the 607 two drugs could potentially allow effective tumor eradication.

608 4.4. Antiangiogenic and antimetastatic effects of allicin

609 Metastasis formation is one of the most complicated and troubling aspects of cancer disease, 610 as it is responsible for approximately 90% of cancer mortality. Metastasis is a process whereby 611 cancer cells invade surrounding tissues, leave the primary tumor, localize to a distant organ, and 612 divide without control. Tumor cells start this process detaching from the primary tumor and 613 invading the tumor's surrounding spaces. The invasion involves the loss of intercellular junctions, 614 the attachment to matrix components, the degradation of the extracellular matrix (ECM), and the 615 actual migration. Blood and lymph will act as dissemination routes for the tumor cells to reach the 616 different body districts. Upon reaching the new target organ, the tumor cells will extravasate and 617 form colonies resulting in metastasis [120].

Allicin showed anti-migration, -invasion and -angiogenic abilities [121,89] (Figure 6). Allicin delayed the gap closure in a scratch assay performed on HGC27, AGS (61.61 μ M, 48h) [89] and renal RCC9863 cells (308.07 μ M, 48h) [11]. In the two gastric cell lines it also reduced the number of cells able to penetrate a porous membrane in a transwell assay [89]. Thus, it blocked the migration and invasion of tumor cells.

Tumors cannot exceed 1-2 mm in size unless they are vascularized. That is, in fact, the 623 624 maximum distance through which oxygen and nutrients can diffuse from blood vessels. 625 Vascularization is necessary to deliver nutrients and oxygen to tumor cells. In addition, newly 626 formed endothelial cells stimulate tumor cell growth by producing growth factors, while the new 627 vessels ensure access of tumor cells to the bloodstream promoting distant tumor spread. 628 Angiogenesis and lymphangiogenesis represent two distinct and complex phenomena, controlled 629 by a delicate balance between factors with proangiogenic activity and factors with antiangiogenic 630 activity. Both phenomena are strictly linked to metastasis and cancer dissemination. The vascular 631 endothelial growth factors (VEGFs) include several mediators with proangiogenic activity: VEGF-A, 632 VEGF-B, VEGF-C, VEGF-D, and PIGF (placental growth factor) 3. VEGFs have multiple proangiogenic

633 activities. They induce the expression of proteases in endothelial cells, such as matrix 634 metalloproteases (MMPs), which free the cells from anchorage to the ECM, allowing their migration. 635 ECM proteins are indeed crucial for the building of new vessels. They also promote the assembly of 636 these new structures from the pre-existing endothelial vasculature surrounding the solid tumor. 637 Endothelial cells (EC) move to the site of the future vessel. There, they proliferate and organize in 638 tubule-like structures and develop the final conformation by recruitment of pericytes and muscle 639 cells [122]. In addition, VEGFs promote the mobilization of hematopoietic stem cells from the 640 marrow, the activation of osteoblasts, the chemotaxis of various immune system cells, and the 641 regulation of neuronal homeostasis. VEGF-A and -B represent key mediators in blood vessel 642 formation; on the other hand, VEGF-C and -D predominantly regulate new lymphatic vessel 643 formation [122].

644 Allicin 1 μ M suppressed EC proliferation induced by basic fibroblast growth factor (bFGF) 645 without affecting normal, unstimulated EC. It inhibited the formation of ECs tubules and intracellular 646 cytoskeleton organization by blocking VEGF-induced actin polymerization. As a comprehensive 647 effect, at the same concentration, allicin counteracted VEGF-bFGF-induced angiogenesis in an aortic 648 ring model, showing a factual antimetastatic potential [121]. HIF1 α is also involved in angiogenesis. 649 Indeed, VEGF is one of the genes that HIF1α modulates in response to hypoxia. On renal cells, allicin 650 (308.07 μ M, 48h) showed a protective role by opposing to the increase in overexpressed HIF1 α and 651 VEGF, confirming antiangiogenic potential [11] (Figure 6).

Allicin 10 μM inhibited both angiogenesis and lymphangiogenesis. *In vitro* and *in vivo*, it blocked VEGF-C-mediated tube formation and reduced the infiltration of ECs and leukocytes. In addition, *in vitro*, it blocked lymphatic HMVEC-dLy cell migration [123]. During the epithelialmesenchymal transition (EMT), epithelial cells lose their phenotype and acquire the characteristics of mesenchymal cells [124]. Thus, carcinoma cells in the EMT stage can invade and metastasize,

allowing tumor progression. Changes in the EMT occur through an orchestrated series of events in which cell-cell and cell-ECM interactions are altered, epithelial cells are released into the surrounding tissue, the actin cytoskeleton is reorganized to allow migration through the ECM, and a new transcriptional program is activated that helps tumor cells to maintain the mesenchymal phenotype [124]. On cholangiocarcinoma cells, allicin 10 and 20 µM inhibited two EMT markers (MMP-2 and MMP-9) and vimentin expression, and upregulated the epithelial marker E-cadherin with an overall effect of blocking invasion and migration [5] (Figure 6).



665 **Figure 6.** Allicin-mediated anti-angiogenic and -metastatic pathways. Created with <u>BioRender.com</u>.

666 **5. In vivo studies**

667 A few studies confirmed allicin antitumor potential *in vivo*. Allicin can counteract tumor 668 growth *in vivo* in different tumor mice models, such as cholangiocarcinoma [5], colon cancer [13], 669 lymphoma (table 2) [4]. As for the *in vitro* experiments, the actual mechanism of action has not been 670 fully elucidated. In cholangiocarcinoma [5] and colorectal [13] tumor models, allicin was able to

671 reduce the tumor burden and at the same time to modulate STAT3 (table 2). In lymphoma-bearing 672 mice, the antagonizing effect on tumor growth of allicin is witnessed by apoptosis [4]. Similarly, on 673 mice xenografted with hepatocellular carcinoma cells, intratumor injection of a mild dose of allicin 674 (500 µg/mouse every other day in consecutive 14 days) suppressed tumor growth inducing both 675 intrinsic and extrinsic apoptosis, partially increasing Bax and FASL mRNA levels to a greater extent 676 than the positive control DOX (20 µg/mouse) [125]. Full disclosure, the best outcome in terms of 677 both tumor growth and apoptosis marker expression comes from the combination of allicin 10 μ g + 678 DOX 20 µg [125], showing the ability of allicin to be used as an adjuvant compound.

679 On B16 and MCA105 xenografted mice, it was clear the importance of the administration 680 pattern. Indeed, on both tumor models, allicin was effective in killing secondary lung tumors at 681 multiple doses and not at the high doses, since at high doses (from 10 mg/kg for the B16 model or 682 25 mg/kg for the MCA105 ones), allicin was less or not effective (table 2) [3]. This behavior may be 683 due to the fact that allicin does not directly affect the tumor, but the immune system mediates its 684 antineoplastic effect. Otherwise, allicin should not reach a plateau but should induce a dosedependent response [3]. To support this assumption, a different study showed that in CH3 mice 685 686 xenografted with bladder MBT-2 cells, 25 µg allicin significantly delayed tumor growth and attracted 687 macrophages, lymphocytes, and fibroblasts to the tumor sites, which represent different markers 688 of immune system activation [17].

A 60-year-old study showed different *in vivo* behavior of allicin. It showed that allicin until 700 mg/mouse/day for 7 days was not able to inhibit tumor growth on mice xenografted with Ehrlich-2 ascites carcinoma or sarcoma 180 cells, while if tumor cells were pre-treated with allicin and then injected in the mice, a carcinolytic effect occurred [126]. The lack of activity cannot even be ascribed to an unsuitable regimen of allicin administration since allicin has been dispensed both as a single dose and repeatedly, using a wide interval of concentration, and through different administration

routes (oral, intraperitoneal and intravenous). Thus, Ehrlich-2 ascites carcinoma or sarcoma 180
cells seems resistant to allicin *in vivo* [126].

697 An interesting study analyzed the effect on the formation of breast cancer metastasis of oral 698 allicin integration (50 mg/kg/day, 5 weeks) to a Mediterranean diet compared to the same 699 integration to a restricted diet. Briefly, mice were fed with different diets: normal rat diet (NRD), 700 Mediterranean (MD), restricted (RD), or a mix of these two diets (MD + RD in a 7:3 ratio). For each 701 group, mice were also orally supplemented with allicin. The overall effect is that allicin alone and 702 combined with all the feeding regimens counteracted the primary breast cancer tumor growth and 703 metastasis formation, limited the circulation of tumor cells, and the development of secondary lung 704 tumors. However, many of these effects were also recorded for the control groups of mice under 705 MD, RD, or MD + RD patterns alone. For instance, allicin and dietary patterns, alone or in 706 combination, inhibited primary tumor volume compared with NRD control, but all allicin groups' 707 effect was not more significant than the only MD or RD groups. Moreover, allicin + RD significantly 708 reduced the number of tumor circulating cells, but there was no significant difference between allicin + RD and allicin or RD alone. However, in contrast to MD or RD alone or in combination with 709 710 allicin, allicin alone did not affect any health marker of mice, such as body weight, suggesting a non-711 toxic effect of this compound when used alone. These results demonstrate that more than allicin 712 alone or combined with the different food regimens, the food regimens themselves have a 713 beneficial effect on primary and secondary tumor formation, showing a higher impact than allicin 714 [127]. To argue in favor of allicin, this compound in this experimental setting was administered orally 715 and probably partially metabolically inactivated. At the same time, allicin was effective in 716 counteracting the toxicity of the diet regimens. Thus, it would be interesting to repeat this study 717 administrating allicin systemically to check if, in that way, the combination of diet and allicin could 718 improve.

On the whole, these results disclosed the antitumor efficacy of allicin *in vivo* in fighting different neoplasms. Only few studies have monitored the safety of allicin use, showing a very favorable profile of allicin and encouraging further studies to confirm this promising outcome.

722

6. Adjuvant antitumor effect of allicin

723 Combination therapy is a pharmaceutical regimen that foresees the use of more than one 724 drug to cure a disease and obtain higher response rates compared to the single treatment. For 725 instance, the synergistic activity of two antineoplastic agents allows to reach the maximum 726 antitumor effect using a lower concentration of each compound and thus to limit the probability of 727 inducing toxicity. This strategy is also common to overcome cancer chemoresistance that can arise 728 from monotherapy or improve single compounds' pharmacokinetics. In particular, allicin has been 729 shown to improve the overall cytotoxic effect of other antitumor agents, limit their toxicity, reverse 730 chemoresistance, or ameliorate their bioavailability.

731

6.1 Improvement of chemotherapy cytotoxicity

The first example is the synergistic activity recorded with 5-fluorouracil (5-FU) on hepatocellular [128], colorectal, and lung [129] cancer cells. Allicin increased the growth inhibition potential of 5-FU and amplified apoptosis both *in vitro* (table 3) (BEL-7402, SK-Hep-1, DLD-1, and SK-MES-1) and *in vivo* (SK-Hep-1 xenografted mice) (table 4), indicating synergism [128].

Engaging the immune system in the fight against cancer represents one of the most successful strategies conceived so far. Many different types of immunotherapies have been developed, and the impressive positive outcomes demonstrate that the rise of an immune response is very favorable not only to eradicate tumors but also to avoid chemoresistance and relapses [130]. IL-2 is a potent chemokine mainly produced by CD4⁺T cells, enhancing the killing activity of CD8⁺T and

741 natural killer (NK) cells. Allicin has immunomodulatory properties, as demonstrated by the increase 742 in NK activity and production of IL-2 [131,132]. Like many other cancer types, pancreatic cancer is 743 characterized by a depressed adaptive and innate immune system [133,134]. In a mice model of 744 pancreatic cancer, the combination of allicin and recombinant IL-2 was tested, showing encouraging 745 results. The combinatory treatment promoted the suppression of tumor growth, prolonged the 746 survival time of mice, and significantly increased CD4⁺T, CD8⁺T, and NK cell infiltration and serum 747 IFN-y levels, reversing the immunosuppressant nature of the tumor microenvironment [132]. Given 748 these outstanding and solid results (high number of mice per group and reliable methods), in the 749 future, it will be interesting to perform prophylactic vaccination experiments matching allicin and 750 IL-2 to understand if the antitumor and immunostimulant potential is enough to induce a vaccine-751 like effect.

In another study, allicin restored normal CD3⁺ and CD4⁺/CD8⁺ T cell *ratio* in mice xenografted with neuroblastoma cells when combined with cyclosporine, suggesting the combination of drugs re-establish immune system homeostasis. The overall effect of the combinatorial drug system was an improved survival compared to mice treated with only cyclosporine (only allicin was not tested) (table 4) [109].

Allicin has been tested *in vitro* and *in vivo* with another natural compound having antitumor activity, artesunate. The association of these two molecules increased the cytotoxic effect of each single agent alone on different osteosarcoma cell lines (MG-63, U20S, 143-B, SaOS-2, and HOS) (table 3). On MG-63 and U20S, allicin + artesunate ameliorated the proapoptotic activity and limited the ability of tumor cells to migrate. *In vivo* experiments confirmed the interesting effects of this combination, and allicin + artesunate suppressed tumor growth to a higher degree than single treatments (table 4) [135].

764 **6.2 Limitation of chemotherapy toxicity**

765 Toxicity and chemoresistance often limit the use of cisplatin in clinical practice. More 766 specifically, in hypoxic conditions, cisplatin can lose efficacy and not be as efficient in killing tumor 767 cells as in a normoxic microenvironment. Allicin synergized the effect of low concentrations of 768 cisplatin in inducing autophagy-mediated cell death in thyroid SW1736 and HTh-7 cancer cells [136] 769 and apoptosis in lung A549 cancer cells in both normoxic and hypoxic conditions at much lower 770 concentrations than cisplatin or allicin alone (table 3) [137]. If in tumor cells allicin enhances the 771 cytotoxic potential, on normal ones, it counteracts it, limiting the toxicity induced by cisplatin. One 772 severe adverse effect of cisplatin is the toxicity on the stria vascularis, which manifests as 773 damage/cytotoxicity of the vestibular hair cells and impairments to the cochlea, which is the cause 774 of the hearing loss that 80% of cisplatin-treated patients experience [138]. On a mice model of 775 cisplatin-induced stria vascularis damage, allicin prevented the formation of the lesions by 776 negatively modulating different cell death mediators, such as caspase 3, PARP-1, and AIF [139]. 777 Therefore, allicin may be used to enhance cisplatin antitumor potential and to limit its debilitating 778 toxicity.

779 Another chemotherapeutic agent widely used in the clinic to treat different tumors but 780 particularly toxic is DOX. In a mouse model of hepatocellular carcinoma, low-dose allicin sensitized 781 mice to DOX, promoting a more potent and safer antitumor effect compared to the effect of DOX 782 alone. The combination provoked the decrease in tumor growth without affecting total body 783 weight. At a molecular level, allicin + DOX promoted apoptosis, modulating both the intrinsic and 784 the extrinsic pathway (table 4) [125]. The dark side of DOX, for its part, is the induction of 785 cardiotoxicity, which very often limits its clinical use [140]. Orally administration of allicin brought 786 into line all examined inflammation and oxidative markers to physiological levels (table 4) and 787 restored normal cardiac morphology and architecture after DOX treatment [141].

Tamoxifen (TAM) is another powerful but toxic anticancer drug widely used to treat hormonedependent breast cancer. One of the many adverse effects induced by TAM is liver injury. Allicin increased TAM's antitumor potential *in vitro* on Ehrlich ascites carcinoma (EAC) cells (table 3), and *in vivo* in a mouse model of EAC (table 4). In the experimental animals, allicin also counteracted the liver injury induced by TAM preventing oxidative stress, lipid peroxidation, and hepatic inflammation and boosting the activity of antioxidant enzymes [142].

794

6.3 Radio- and chemoresistance reversion

795 Allicin showed a radio-sensitization effect on different glioma and colon cancer models 796 [15,101]. On U87MG glioma cells, allicin allowed sub-toxic doses of radiation to become cytotoxic 797 through the induction of DNA damage [101]. On its part, colon cancer is particularly prone to acquire 798 resistance to radiations, which is usually related to the abnormal expression of NF-KB signaling 799 pathway. The combination of radiation and allicin not only increased the apoptotic and anti-800 migration ability of radiations on HCT116 cells but also increased its potential in vivo on mice 801 xenografted with CT26 cells. Both *in vitro* and *in vivo*, allicin combined with X-ray radiotherapy 802 downregulated NF-κB and IKKβ (both phosphorylated and not-phosphorylated form), while 803 promoted $I \kappa B \alpha$ phosphorylation [15].

The tumor suppressor miR-486 is often downregulated in different tumors, such as glioblastoma, and can cause chemoresistance. miR-486 directly binds O⁶-methylguanine-DNA methyltransferase (MGMT), an enzyme that actively promotes DNA repair. Basically, MGMT protects cells from alkylating agents. If these cells are tumor ones and the alkylating agent a chemotherapy one, it is easy to understand that its overexpression promotes the rise of resistance, and its antagonization represents an antitumor strategy [143], [144]. Glioblastoma cells usually develop resistance to temozolomide in this way. Both *in vitro* and *in vivo*, allicin was able to sensitize

811 resistant glioblastoma cells/tumors to temozolomide by increasing miR-486-3p and decreasing
812 MGMT expression [143].

813 Resistance to antineoplastic therapy and relapses are often due to the presence of cancer 814 stem cells (CSC) and the so-called "side population" (SP). The SP contains multipotent stem cells 815 characterized by high tumor-initiating ability. For instance, SP isolated from multiple myeloma is 816 resistant to dexamethasone (DX), the gold standard for multiple myeloma treatment, and 817 represents one of the most severe causes of therapy inefficacy [145,146]. However, adding allicin 818 to DX inhibited the proliferation of SP sorted from two myeloma cell lines, RPMI-8226 and NCI-H929. 819 The drug combination promoted a cell-cycle arrest in the G1 phase and apoptosis. As already 820 reported, allicin can modulate different miRNAs. In this case, allicin + DX was able to increase the 821 expression of miR-127-3p, which is a tumor suppressor and is often downregulated in different tumor types [135,143,147], such as myeloma [148–150]. The effect of the combinatorial treatment 822 823 on miR-127-3p is essential to inhibit proliferation and induce apoptosis and may be associated with 824 the inhibition of the PI3K/AKT/mTOR (phosphoinositide-3-kinase/serine/threonine protein kinase 825 B/mechanistic target of rapamycin) pathway [148], known to control cell survival and often 826 overexpressed in several cancer types [151]. Accordingly, the inactivation of that miRNA revoked 827 the ability of allicin and DX to inhibit the PI3K/AKT/mTOR signal cascade and, at the cellular level, 828 the cytotoxic and antiproliferative effect [148].

5-FU is approved for the treatment of gastric carcinoma, but chemoresistance limits its application. The anticancer effects of allicin, 5-FU, and allicin/5-FU on the 5-FU resistant gastric MKN-45 cancer cells were evaluated by MTT assay and DAPI staining. The expression of the Pglycoprotein (P-gp) and CD44 proteins was also determined using immunocytochemistry. The combination of allicin with 5-FU significantly increased apoptosis and decreased the expression of the P-gp and CD44 proteins compared to 5-FU alone [152].

835 The simultaneous eradication of tumor cells and thus CSC guarantees an efficient and 836 exhaustive antitumor effect, which should also limit relapses. In melanoma cells, CD44⁺ and CD177⁺ 837 have been characterized as CSC-like cells. High levels of those cells are correlated with increased 838 metastatic risk and decreased survival rate [153–155]. One of the most effective drugs used to cure 839 malignant melanoma is all-trans retinoic acid (ATRA). Unfortunately, its antitumor potential is 840 hampered by chemoresistance due to CD44⁺ and CD177⁺ populations. Very interestingly, especially 841 on CD44⁺ melanoma cells, subtoxic concentrations of allicin (5 μ g/mL – 30.08 μ M) were able to 842 restore ATRA sensitivity and enhance its antitumor potential. First, allicin enhances the ability of 843 ATRA to reduce CD44⁺ and CD177⁺ subpopulation frequency on the heterogeneous melanoma 844 population (A375 cells). Specifically, on the single resistant cell types, allicin increased ATRA 845 cytotoxic and antiproliferative effect, and on CD177⁺ also its anti-progression effect by decreasing 846 MMP-9 protein expression (table 3) [156].

847

6.4 Improvement of chemotherapy bioavailability

848 A different strategy to combine the effect of two drugs is to synthesize analogs bearing the 849 two active moieties in one single compound. In this way, each moiety can contribute to improve the 850 drug efficiency, in terms of potency or pharmacokinetics. In addition, projecting the use of the 851 hypothetical molecule clinically, the use of one single drug instead of two will be better accepted by 852 the patients. Bearing in mind that often oncological patients face therapeutic regimen hard to follow 853 in terms of the number of medicines and timing, the use of a single drug would probably ameliorate 854 the therapy compliance. 6-Mercaptopurine (6-MP) and 6-MP-riboside (6-MPR) are SH-containing 855 purine analogs with antileukemic potential. However, they are characterized by low availability. On 856 its side, allicin is not stable at room temperature and is fast metabolized, but it easily penetrates 857 cells. Miron et al. created two purine analogs linking 6-MP or 6-MPR to allicin. In this way, the

858 performance of all compounds improved. Indeed, S-allyl-6MP (SA-6MP) and S-allyl-6-MPR (SA-859 6MPR) showed good bioavailability and chemical stability. The antileukemic effect of these two 860 derivatives has been tested on primary B-chronic lymphoblastic leukemia (CLL) cells in vitro and in 861 vivo. SA-6MP and SA-6MPR killed tumor cells and induced apoptosis on ex vivo samples and animal 862 tumor models. The two new analogs showed almost the same effects in the same entity, which were 863 significantly improved compared to 6MP and 6MPR alone. Allicin alone was not tested. The Authors 864 of the study hypothesize that the so promising antileukemic effect was due to the ability of allicin 865 to deplete GSH [157]. Indeed, B-CLL lymphocytes are characterized by high levels of this protein 866 (twice as high as those in B-lymphocytes from healthy subjects) and were very sensitive to GSH 867 depletion [158]. However, in the study, they did not check the GSH levels or the effect on GSH of 868 SA-6MP and SA-6MPR, and this plausible postulate has still to be verified [157].

869

7. Selectivity towards tumor cells

The positive anticancer potential of a compound is the result of a favorable benefit/risk *ratio*. Thus, such a compound must be able to eradicate tumors together with a negligible toxic effect. The lack of selectivity towards tumor cells is one of the main reasons of the occurrence of toxicity; thus, it represents a useful information to predict the outcome of *in vivo* studies.

Although it is a common belief that natural compounds, given their organic nature, are considered safe, the vast number of natural anticancer drugs which induce toxic effects clearly prove the opposite. In the case of antitumor drugs, the toxicity that causes chemotherapy's side effects is linked to a lack of selectivity of action towards tumor cells.

Allicin was tested on primary foreskin human fibroblasts obtained from three different individuals. In this test, two out of the three derived cell lines were sensitive to allicin, which inhibited cell proliferation with IC_{50} (half maximal inhibitory concentration) close to 16 and 40 μ M,

respectively. Besides, the third one did not respond to allicin at all. In this study, as a reference, human mammary MCF7, endometrial Ishikawa, and colon HT-29 cancer cells showed to be more sensitive than the *ex vivo* fibroblasts since the IC₅₀ were all comprised between 10 and 25 μ M. A similar outcome happened testing allicin on lymphoma L5178Y and normal spleen cells: IC₅₀ on tumor cells was exactly 2.46 times smaller than that recorded for spleen cells (443.62 μ M *versus* 1.09 mM, respectively) [4].

887 Conversely, allicin was not safe for blood cells. 10 µM of this compound promoted apoptosis 888 of THP-1 monocytic leukemia cells but also hemolysis and eryptosis, showing a null selectivity of 889 action towards tumor cells. In particular, in the red blood cells, allicin promoted p38-MAPK-890 mediated intrinsic apoptosis [63]. These results unravel a limit in the use of allicin. This sole datum 891 suggests that allicin use is precluded from treating liquid tumors and raises questions about the 892 systemic use of this compound. Anemia may develop as a consequence of toxicity to red blood cells 893 and preclude the use in patients. However, further studies are needed to confirm this outcome in 894 vivo.

895 GSH oxidation has been shown as crucial in allicin-induced apoptosis in different tumor cell 896 lines, but it is also probably connected with the high toxicity on normal cells. Indeed, as a double-897 edged sword, and since many vital proteins and enzymes have easily accessible cysteine groups, the 898 interaction with allicin might explain its cytotoxic potential and toxicity on normal cells. Normal 899 epithelial HUVEC cells were more sensitive to allicin treatment than different tumor or immortalized 900 cells used as a reference: human adenocarcinoma A549, immortalized mouse 3T3 fibroblasts, and 901 human mammary carcinoma (MCF7) cells. Allicin promoted approximately 50% of cell death of 902 HUVEC cells at 9.4–18.8 µM, while for all other cell lines the same effect was reached in the interval 903 $37.5 - 188 \,\mu$ M. The difference in the potency of allicin could be explained by the different impact of 904 the GSH modulation in normal and tumor cell lines. HUVEC cells showed a higher sensitivity to GSH

905 depletion and, conversely to 3T3 and MCF7, the authors of the study claimed that HUVEC died 906 accidentally (necrosis) instead of apoptosis. Of note, oddly, in the same study, allicin seemed to 907 promote necrosis also on HT29 and A549 cells [91]. However, the methods described in the study 908 to distinguish between apoptosis and necrosis could be misleading. Indeed, to assess apoptosis, 909 cells were treated for 30 or 60 minutes. The probe used to detect apoptosis (YOPRO-1 iodide) 910 showed medium/late apoptotic events (nuclear condensation and DNA fragmentation). Thus, the 911 induction of necrosis reported after allicin treatment relies on the lack of morphological events 912 recorded at a very early time points but looked at medium/late endpoints. Furthermore, cell death 913 kinetics can be different between cell and tumor types. Thus, the morphological event highlighted 914 by YOPRO-1 iodide in allicin-treated 3T3 and MCF7 could happen later in HUVEC, HT29, and A549. 915 The authors did not test more prolonged treatment with allicin and directly assumed that necrosis 916 occurred instead of apoptosis. Instead of increasing the incubation time with allicin, they increased 917 the concentrations. Only at very high concentrations (starting at 75 µM for the three cell lines) and 918 immediately reaching a plateau, a slight non-significant increase in YOPRO-1 iodide positive cells 919 occurred. Oddly, the entity of the effect of allicin 75 µM on the three cell lines was precisely the 920 same, even though the MTT test at 24h showed different sensitivity to the compound. Thus, if we 921 add that allicin promoted apoptosis in HT29 [14,91] and A549 [109] cells in different studies at 922 similar concentrations, further data will be needed to confirm the accidental cell death and support 923 the overall discovery of the study.

Overall, the results outlined above are not conclusive and show a different sensitivity to allicin dependent on the cell type. Anyhow, allicin seems to hold a favorable antitumor profile in the big picture, especially given the possibility of exploiting the above-mentioned "in-situ strategy". Indeed, as seen in paragraph 3.1, the in-situ generation of allicin from alliinase could come to the rescue to overcome the potential toxicity. For instance, in the lack of selectivity towards THP-1 cells

associated with hemolysis and eryptosis [63], a marker for monocytes could be exploited to deliver
alliinase specifically on tumor cells and avoid a broad range effect involving all blood cells. Indeed,
for both solid and liquid tumors, the latter tumor-selective strategy improved the anticancer efficacy
and circumvented allicin's impact on the non-target cells, increasing the efficiency and the safety of
the treatment [59,62].

934 **8.** Conclusions

Gancer is a leading cause of death globally [159]. Consequently, finding novel therapeutic
strategies and less toxic natural-based compounds are mandatory for the treatment of patients.
Allicin is a natural product widely consumed in most cultures being one of the active garlic
compounds with multiple potential health benefits [1].

939 Over the past years, allicin has been broadly employed due to the antimicrobial, anti-940 inflammatory, antioxidant, immunomodulatory effects, and cardiovascular protection properties. 941 In the last few decades, the anticancer activity of allicin has kindled the interest of health 942 professionals worldwide. Cancer is a multi-factorial disease including alterations in the cell signaling 943 pathways. Allicin has multiple cellular targets in human cells and is able to inhibit cell proliferation, 944 angiogenesis and metastatic process, increase the expression of tumor suppressor genes, induce 945 apoptosis, and modulate various other genetic pathways, demonstrating interesting anticancer 946 properties, especially in vitro [1]. Moreover, allicin has been widely studied in combination with 947 cancer chemotherapy. Currently, the combination of anticancer drugs exhibiting different 948 mechanisms of action is widely used in the oncological field to improve anticancer drugs' 949 pharmacotoxicological profile. Many studies suggest that plant extracts and phytochemicals like 950 allicin, in combination with anticancer drugs, can potentiate the anticancer effects and cytotoxicity 951 of anticancer drugs and limit their toxicity, as reported above. However, even if the interactions

between phytochemicals and traditional anticancer drugs could be clinically advantageous, the type
of interaction needs to be carefully explored in animal models and clinical trials to exclude
toxicologically relevant reactions.

955 Among the most relevant interactions, the pharmacokinetic ones (and the metabolic ones in 956 particular) certainly stand out. Allicin in vitro inhibited cytochrome P450 (CYP) 1A2, CYP3A4 and CYP 957 2C19 activity [160,161]. However, to date there are conflicting in vitro results on garlic effect on 958 CYP450 and data from clinical studies show how garlic supplements decrease, rather than increase, 959 plasma concentrations of CYP3A4 substrates such as saquinavir [162–164], probably due to the 960 induction of CYP3A4 in gut mucosa. The ability of a compound to modulate the enzymes of drug 961 metabolism positively or negatively is reflected to a greater or a lesser biotransformation of the 962 drugs simultaneously administered. Even if partial, and sometimes conflicting, this clinical evidence 963 suggests that allicin consumption might represent a potential risk for patients receiving 964 polypharmacy. The associated risks are therapeutic failure, overdose and adverse events related to 965 it. For this reason, the risks and adverse events of allicin should be cautiously considered. Moreover, 966 the high instability, reactivity, and volatility of allicin remain a limitation for its future applications 967 and future efforts should be directed to allicin pharmaceutical formulation.

968 It is just the low chemical stability of allicin that may partly explain the lack of its clinical 969 application. As we pointed out above, the data on pharmacokinetic and also on the metabolic 970 pathways are evidently still few. Despite the remarkable in vivo antitumor effects of allicin on 971 several cancer types and models and its ability to act on different molecular targets, the preclinical 972 studies were not confirmed by the same number of clinical ones [20]. On clinical trials.gov, we found 973 only one trial addressing the application of allicin in follicular lymphoma (NCT00455416), but no 974 data are published yet; in addition, we found a further trial on Pubmed, referring to the effects of 975 local application of allicin on progressive gastric carcinoma. Allicin was infused via gastroscopy to

976 the lesion region of 40 patients 48h before gastrectomy. It was observed that allicin could inhibit 977 cell growth and proliferation and promote cell apoptosis upregulating the protein expression of Bax 978 and Fas and downregulating that of Bcl-2 in the gastric carcinoma tissue [165].

In contrast, many human studies were conducted with garlic. Among these, a double-blind, randomized clinical trial on patients with colorectal adenomas has shown that aged garlic extract (AGE) suppresses cancer progression [166]. A blinded randomized controlled trial reported that daily administration of garlic oil and garlic extract for 7 years significantly decreased risk of death due to gastric cancer for more than 22 years [167].

Regarding the epidemiological evidence, the major studies show protective effects of garlic against gastro-intestinal cancer. The most recent meta-analysis was conducted on a total of 8,621 cases and 14,889 controls: garlic intake was associated with reduced risk of gastric cancer [168]. The findings on colorectal cancer are controversial. Based on a recent meta-analysis including 11 studies and involving 12,558 cases, garlic intake could reduce the risk of colorectal cancer [169], but this was not confirmed by previous epidemiological research [170,171].

990 In conclusion, more studies on the antineoplastic effects of allicin would allow a better 991 understanding of its pharmacological mechanisms of action that can be useful in discovering the 992 therapeutic potential of allicin, both alone and in association with standard anticancer 993 chemotherapy. However, despite the presumed safety of natural compounds, more investigations 994 on the toxicity of allicin are needed to determine possible adverse events and the optimum 995 therapeutic dosage. Moreover, careful tailored pharmacokinetic studies are also required for the 996 biopharmaceutical development of allicin appropriate products. Proper formulations with an 997 acceptable stability and bioavailability are needed to facilitate clinical applications.

998

Table 1. Anticancer effect of allicin in vitro

Cell type	Concentration and duration	Antitumor effect	Molecular mechanisms, if	Reference
MCF-7	28,51 μM - 40 μM, 24h	Citotoxicity, apoptosis	↓ ΔΨm ↑ Caspase 3 ↑ Caspase 8 ↑ NOXA ↑ p21 ↑ BAK ↓ BCL-XL	[7,87]
	37.5 – 188 μM 30min - 24h		↓ GSH ↑ ROS	[91]
	41.23 μmol/L	Cytotoxicity	Not reported	[8]
HCC-70	20 μM, 24h	Apoptosis	 ↓ ΔΨm ↑ Caspase 3 ↑ Caspase 8 ↑ NOXA ↑ p21 ↑ BAK ↓ BCL-XL 	[87]
AGS	10 μg/mL, 48 h	Cytotoxicity, apoptosis, migration inhibition	↑ Bax ↓ Bcl-2 ↑ miR-383-5p ↓ ERBB4	[89]
	500-1000 μM, 48 h	Apoptosis, proliferation inhibition	G2/M cell-cycle arrest 个 Phospho-Histone3	[90]
	10-40 μM	Cytotoxicity	Not reported	[9]
H1-29	37.5-70 μM, 24h	Cytotoxicity	↓ GSH 个 ROS	[91]
	10 μg/ml, 24 - 72h	Cytotoxicity	Not reported	[14]
	10 μg/ml, 24 - 72 h	Cytotoxicity	Not reported	[14]
Caco2	500-1000 μM, 48 h	Apoptosis, proliferation inhibition	$\downarrow \Delta \Psi m$ G2/M cell-cycle arrest \downarrow GSH	[90]
HepG2	19,29 μM, 24 h	Cyotoxicity	not reported	[7]
HepG2 p53 wt	35 μM, 3-48 h	Autophagy	 ↑ Beclin-1 ↑ Bad ↑ p-AMPK ↑ TSC2 ↑ Atg7 ↓ PI3K/ mTOR ↓ p-Bcl-2 ↓ Bcl-xL ↓ p53 	[100,172]
HepG2 p53 silenced	35 μM, 24h	Apoptosis	↑ ROS ↑ Bax ↓ Bcl-2	[100]

			个 AIF 个 EndoG	
			个 Htra2/omi	
			↑ Caspase 3	
			↑ Caspase 8	
			个 Caspase 9	
			↓ ΔΨm	
			个 ROS	
			个 Bax	
			↓ Bcl-2	
			个 AIF	
Hen2D nF2 mutation	25M 24b	Anontosis	个 EndoG	[100]
нерзв рэз питатоп	35 μiνi, 241	Apoptosis	个 Htra2/omi	[100]
			个 Caspase 3	
			个 Caspase 8	
			个 Caspase 9	
			$\downarrow \Delta \Psi m$	
PC-3	77,92 μM, 24h	Cyotoxicity	Not reported	[7]
		, ,	个 PARP	
SK-N-FI		citotoxicity, apoptosis	Ornithine Decarboxylase	
	IC50 72 h: 18.6 μM		Inhibition	[116]
			个 PARP	[110]
SK-N-AS	IC50 72 h: 19 48 uM	citotoxicity apontosis	Ornithine Decarboxylase	
SK N AS	1030 / 2 Π. 13,40 μινι		Inhibition	[116]
				[110]
SK N Ro(2)c	IC50 72 b: 10 27 uM		Ornithing Decarboxylase	
SK-N-Be(2)c	10,27 μινι	citotoxicity, apoptosis		[116]
				[110]
Kolly	ICE0 72 b: 0.21 uM		FARF	
Keny	1050 72 11. 9,21 μίνι	citotoxicity, apoptosis		[116]
				[110]
1107040	00 14 24	A		[00]
U87MG	90 μM, 24n	Apoptosis	1` p38	[96]
			1 p53	
HMCV-transfected U87MG	60 μg/mL, 24-168h	Cytotoxicity	↓IL-6	[101]
			ψIFN-β	[101]
			个 Bax	
Tca-8113	12.5 - 50 μg/mL , 24-48 h	Apoptosis	↓ Bcl-2	
			个 Caspase 3	
				[86]
			个 Bax	
SCC-25	12.5 - 50 μg/mL 24-48 h	Apoptosis	↓ Bcl-2	
			↑ Caspase 3	[86]
SGC-7901	15 - 120 µg/ml, 24-72h	Apontosis	↑ Cytochrome c release	[84]
55507551	20 220 μβ/mb, 21 / 2m	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	↑ Caspase 3	[01]

			 ↑ Caspase 8 ↑ Caspase 9 ↑ Bax expression ↑ Fas expression 	
	0.5-10 μg/mL, 12-48 h	Cytotoxicity, apoptosis	 ↑ Cleaved caspase 3 ↑ p38-mapk expression 	[106]
	0,016 - 0,1 mg/mL, 12-48h	Apoptosis, proliferation inhibition	G2/M cell-cycle arrest ↓ Telomerase activity	[6]
	3 mg/L, 12 - 48 h	Cytotoxicity, proliferation inibition	G2/M cell-cycle arrest	[85]
HGC27	10 µg/mL, 48 h	Cytotoxicity, apoptosis, migration inhibition	↑ Bax ↓ Bcl-2 ↑ miR-383-5p ↓ ERBB4	[89]
SiHa	1 - 50 μM, 24h	Apoptosis	 ↑ DNA condensation ↑ Apoptotic bodies ↑ DNA fragmentation ↑ Caspase 3 ↑ Caspase 9 ↑ PARP 	[88]
L929	50 μΜ	Apoptosis	个 Apoptotic bodies 个 DNA fragmentation	[88]
U251	30 - 60 μg/mL, 24h	Apoptosis	↑ FasL ↑ Caspase 3 ↓ Bcl-2	[83]
HuCCT-1 QBC939	10 - 40 μM, 24 - 72h	Apoptosi and EMT	 ↑ Caspase 3 ↑ Caspase 9 ↑ Bax ↓ Bcl-2 ↓ MMP-2 ↓ MMP-9 ↓ vimentin ↑ cadherin E 	[5]
SKOV3	25 μg/mL, 12- 48h	Apoptosis	 ↑ Bax (citosolic fraction) ↓ Bax (mitochondria fraction) ↑ Cytocrome c (citosolic fraction) ↓ Cytocrome c (mitochondrial fraction) ↑ pJNK/JNK 	[108]
RCC-9863	308.07 μM, 48h	Apoptosis, anti-migration effect	↓ HIF-α ↓ VEGF ↓ Bcl-2	[11]

Sporadic clear cell renal cell carcinoma (ex vivo)	308.07 μM, 48h	Apoptosis, anti-migration effect	↓ HIF-α ↓ VEGF ↓ Bcl-2	[11]
	36 μM, 24 h		not reported	[7]
	30 µg/mL, 72h		Not reported	[10]
A549	37.5 – 188 μM, 24h	Cyotoxicity	↓ GSH ↑ ROS	[91]
NIH-3T3	0.5 μM, 30 min	Proliferation inhibition	microtubule depolymerization	[119]
3t3	37.5 – 188 μM 30min - 24h	Cytotoxicity, apoptosis	↓ GSH ↑ ROS	[91]
Huvec	9.4–18.8 μM, 24h	Cytotoxicity	↓ GSH ↑ ROS	[91]
L5178Y	72 μg/mL, 24h	Cytotoxicity, apoptosis	↑ Caspase 3	[4]
THP-1	10 μM, 24h	Cytotoxicity, apoptosis	Not reported	[63]
AGS	5 - 20 μg/mL, 24h	Parthanatos	个 Bax 个 AIF 个 Cytochrome c	[93]
Ishikawa	10-40 μM	Cytotoxicity	Not reported	[9]
DS19	2-5 μM 24-72h; 50 μM 2h	Proliferation inhibition	↑ Histone 4 acetilation	[114]
SK-N-SH	5 μM , 12-48h	Apoptosis	 ↑ Caspase 3 ↑ Caspase 9 ↑ Cytochrome c release ↑ P38-MAPK 	[107]
EL-4	4-8 μg/mL, 24-48 h	Apoptosis	 ↑ Caspase 3 ↑ Caspase 12 ↑ Bax/Bcl-2 ↑ Cytochrome c ↓ ΔΨm 	[92]
MGC-803	0.5-10 µg/mL, 12-48 h	Cytotoxicity	Not reported	[106]
BGC-823	0.5-10 μg/mL, 12-48 h	Cytotoxicity, apoptosis	 ↑ Cleaved caspase 3 ↑ p38-mapk expression 	[106]
LS174T	10 μg/ml, 24 -72 h	Cytotoxicity	Not reported	[14]
	25 μM, 24 h	Apoptosis	↓ pSTAT3 ↓ MCL-1, ↓ Bcl-2 ↓ Bcl-xL	[13]
HCIII6	10 μg/ml, 24 h	Apoptosis, proliferation inhibition	↑ Bax ↓ Bcl-2 ↑ Cytochrome c ↑ Nrf-2	[14]
HL60	5 μM , 16 h	Apoptosis, proliferation inhibition	 ↑ Cytochrome c ↑ Caspase 3 ↑ Caspase 9 ↓ GSH G2/M cell-cycle arrest 	[2]

U937	20 μM, 16 h	Apoptosis, proliferation inhibition	 ↑ Cytochrome c release ↑ Caspase 3 ↑ Caspase 9 	[2]
MBT-2	0.1 - 2.5 mg/mL, 48 h	Cytotoxicity	Not reported	[17]

Abbreviations: AIF (apoptosis-inducing factor); Atg7 (autophagy related 7); Bad (Bcl-2 associated agonist of cell death); Bak (Bcl-2 antagonist/killer); Bax (Bcl-2-like protein 4); Bcl-2 (B-cell lymphoma 2); Bcl-xL (B-cell lymphoma-extra large); ΔΨm (mitochondrial potential); EndoG (Endonuclease G); ERBB4 (Erb-B2 Receptor Tyrosine Kinase 4);); ERK (extracellular signal-regulated kinases); FASL (FAS ligand); GSH (glutathione); HIF,(hypoxia-inducible factor); HtrA2 (high-temperature requirement A2); IFN-β (interferon β); IL-6 (interleukin-6); JNK (c-Jun N-terminal kinase); MAPK (mitogen-activated protein kinase); MCL-1 (mycloid cell leukemia-1); MMP-2 (matrix metalloproteinase 2); MMP-9 (matrix metalloproteinase 9); mTOR (mechanistic target of rapamycin); NOXA (phorbol-12-myristate-13-acetate-induced protein 1); Nrf2 (nuclear factor-erythroid factor 2-related factor 2); p21 (cyclin-dependent kinase inhibitor 1); p-AMPK (phospo-AMP activated protein kinase); PARP (Poly (ADP-ribose) polymerase-1); p-Bcl-2 (phospho-B-cell lymphoma 2); PI3K (phosphoinositide-3kinase); pJNK (phospho c-Jun-N-terminal kinase); PKA (protein kinase A); pSTAT3 (phospo-signal transducer and activator of transcription 3); ROS (reactive oxygen species); SHP-1 (Src homology region 2 domaincontaining phosphatase-1); TSC2 (Tuberous Sclerosis Complex 2); VEGFs (vascular endothelial growth factors).

Table 2. Anticancer effect of allicin in vivo

Mouse model	Dose and duration	Antitumor Effects	Molecular mechanisms, if reported	Reference
Nude athymic mice bearing cholangiocarcinoma xenografts	10 – 20 mg/kg daily for 4 weeks	↓ tumor weight	↑ p-STAT3 levels ↓ Cleaved caspase 9 ↓ Vimentin	[5]
C57BL/6 mice treated to develop colorectal cancer	0.24 mg/day/mice for 100 days	↓ Number of tumors ↓ Tumor size ↓ Average tumor load	Not reported	[13]
BALB/c mice inoculated with L5178Y	20 mg/kg for 7 days	↓ Tumor growth ↑ Survival	Not reported	[4]
BALB/c mice bearing 4T1 xenografts	50 mg/kg/day for 13 weeks	 ↓ Circulating tumor cells ↓ Primary tumor growth and weight 	Not reported	[127]
Athymic female mice bearing 6ES-2- Luc xenografts	Daidzein-alliinase (Dall) conjugate (150 µg) + of alliin 3 mg for 15 days	↓ Tumor growth	Not reported	[60]
CD-1 nude mice bearing N87 xenografts	Anti-ErbB2-alliinase conjugate 20 mg mAb/mouse + alliinase 20 mg/mouse + alliin 6 mg for 2–4 weeks	\downarrow Tumor growth	Not reported	[61]

Human/mouse chimeric BALB/c mice engrafted with human B-CLL PBMC	Conjugate 80 µg/mouse + alliin 3 mg/mouse for 3 days	↓ Tumor growth	Not reported	[62]
C57BL bearing B-16	5 mg/kg for 13 days	↓ Lung metastasis	Not reported	[3]
C57BL bearing MCA-105	12.5 mg/kg for 14 days	↓ Lung metastasis	Not reported	[3]
Swiss mice bearing Ehrilch-2 or Sarcoma 180	16 - 810 μg/kg for 7 days	Carcinolytic effect	Not reported	[126]
BALB/c mice bearing BEL7402 xenograft	1 - 5 mg/ml for 14 days.	↓ Tumor growth	↑ Apoptosis ↑ Bax and FASL mRNA levels	[125]
CH3 mice bearing MBT-2 xenograft	12.5 - 25 µg for 19 days.	\downarrow Tumor growth	Not reported	[17]

Abbreviations: ADM (adriamycin); Erb2 (human epidermal growth factor receptor 2); HCV-ABxtl68 (anti-Hepatitis C Virus human monoclonal antibody); i.p. (intraperitoneal); i.v. intravenous; MD (Mediterranean diet); p-STAT3 (phosphorylated- Signal transducer and activator of transcription 3); PLP (pyridoxal phosphate); RD (restricted diet); s.c (subcutaneous).

Table 3. Adjuvant antitumor effect of allicin in vitro

Cell type	Concentration and duration	Antitumor Effects	Molecular mechanisms, if reported	Reference
A549	Allicin 10 μg/mL + cisplatin 2 μg/mL for 24h	Synergistic apoptosis	Not reported	[137]
SK-Hep-1	Allicin 3 - 10 μg/ml + 5-FU 100 - 300 μg/ml for 48h	Synergistic cytotoxic effect ↑ Apoptosis	 ↑ ROS ↓ Mitochondrial potential ↑ Caspase 3 ↑ PARP ↓ Bcl-2 	[128]
BEL-7402	Allicin 3 - 10 μg/ml + 5-FU 100 - 300 μg/ml for 48h	Synergistic cytotoxic effect ↑ Apoptosis	 ↑ ROS ↓ Mitochondrial potential ↑ Caspase 3 ↑ PARP ↓ Bcl-2 	[128]
DLD-1	Allicin 26.76 μM + 5-FU 107.25 μM for 24h	Caspase-independent cell death	Not reported	[129]
SK-MES-1	Allicin 4.31 μM + 5-FU 101.1 μM for 24h	Apoptosis	Not reported	[129]
143-B, SaOS-2 and HOS	Allicin 5 - 10 μM + Artesunate 50 - 100 μM for 24-72h	↑ Cytotoxicity	Not reported	[135]
Temozolomide resistant U251 (U251- TR)	Allicin 30 $\mu g/mL$ + temozolomide (TMZ) 50 $\mu g/mL$ for 24h	↑ Apoptosis	Not reported	[143]
Ehrlich ascites carcinoma cells (EAC)	Allicin 10 μM + TAM 3 μM for 24h	↑ Cytotoxicity	Not reported	[142]
MG-63 and U20S	Allicin 5 - 10 μM + Artesunate 50 - 100 μM for 24-72h	↓Invasion and motility ↑Cytotoxicity ↑Apoptosis	个Caspase 3/9 expression and activity	[135]

		↓ Colony formation		
Side population sorted from RPMI- 8226 and NCI-H929 cells	Allicin 10 μg/mL + dexamethasone 50 μM 24-72h	↓ Cell proliferation ↑ Apoptosis	↑ G1 cell-cycle arrest ↑ miR-127-3p expression ↓ PI3K ↓ p-AKT/AKT ↓ p-mTOR/mTOR	[148]
HCT-116	Allicin 10 μg/mL + X-ray 4 Gy - 30 minutes for 7 days	↑ Apoptosis	↓ NF-κB ↓ p-NF-κB ↓ ΙΚΚβ ↓ p-ΙΚΚβ ↑ΙκΒα ↑p-ΙκΒα	[15]
CD44+ A375	Allicin 5 μg/mL + ATRA 37.43 μM for 48h	↑ Cytotoxicity	Cell-cycle arrest in S phase 个MMP-9 个Cyclin D	[156]
CD117+ A375	Allicin 5 μg/mL + ATRA 8.09 μM for 48h	↑ Cytotoxicity	↑ RARβ Cell-cycle arrest in S phase ↓ MMP-9 ↑ Cyclin D	[156]
U937	X-ray 10 Gy + allicin 30 - 60 μg/mL for 24 – 48h	↑ Cytotoxicity	个 DNA damage	[101]
SW1736 and HTh-7	Allicin 10 μM + carboplatin 10 μM for 48h Allicin 10 μM + cisplatin 10 μM for 48h Allicin 10 μM + rapamycin 10 μM for 48h	个 Cytotoxicity 个 Autophagy	↑ LC3II ↑ p62	[136]

Abbreviations: 5-FU (5-fluorouracile); AKT (protein kinase B); ALP (alkaline phosphatase); ATRA (all-trans retinoic acid); Bcl-2 (B-cell lymphoma 2); Gy (gray); i.p. (intraperitoneal); IKKβ (inhibitor of nuclear factor kappa-B kinase subunit beta); IkBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha); LC3II (microtubule-associated protein 1A/1B-light chain 3 II); MMP-9 (matrix metallopeptidase 9); mTOR (mechanistic target of rapamycin); NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells);); p-AKT (phospho-protein kinase B); p-IKKβ (inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells);); p-MTOR (phospho-mechanistic target of rapamycin); NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells); p-mTOR (phospho-mechanistic target of rapamycin); p-NF-kB (phospho-nuclear factor kappa-light-chain-enhancer of activated B cells); p62 (Sequestosome-1); PARP (Poly ADP ribose polymerase); PI3K (phosphatidylinositol 3-Kinase); RARβ (retinoic acid receptor beta); TAM (tamoxifen); TMZ (temozolomide).

Table 4. Adjuvant antitumor effect of allicin in vivo

Mouse model	Dose and duration	Antitumor Effects	Molecular mechanisms, if reported	Reference
athymic nude mice bearing SK-Hep-1 xenografts	Allicin 5 mg/kg/day + 5-FU 20 mg/kg/day for 3 weeks	↓ Tumor volume and weight ↑ Apoptosis	Not reported	[128]
C57/BL6 nude mice bearing BXPC-3 xenografts	Allicin 10 mg/kg + rIL-2 20 μg/mL for 28 days	↓ Tumor growth ↑ Overall survival time ↑ CD4+, CD8+ T cells, NK infiltration	Ϋ́INF-γ	[132]
Congenic Immuno-deficient NOD/SCID mice	Allicin 50 mg/kg Allicin + TMZ 60 mg/kg for 3 weeks	↑ Overall survival time	Not reported	[143]

Swiss albino mice bearing Ehrlich ascites carcinoma cells (EAC) xenografts	Allicin 10 mg/kg + TAM 1 mg/kg for 21 days	↓ Tumor growth	 ↓ ALT, AST, gGT, LDH, ALP, and total bilirubin ↓ Lipid peroxides ↑ GSH, and SOD ↓ TNF-α 	[142]
Male Swiss albino mice	Allicin 20 mg/kg + DOX 10 - 20 mg/kg for 2 weeks	↓ Tumor growth	 ↓ AST, LDH, CK and CK- MB ↓ IL-1β, TNF-α, 8-OHdG ↓ Nitric oxide and malonaldehyde ↑GSH, CAT, SOD, GPx ↓ COX2 	[141]
BALB/c mice bearing MG-63 xenografts	Allicin 5 mg/kg/day + artesunate 50 mg/kg/day for 24-72h	↓ Tumor growth	Not reported	[135]
BALB/c mice bearing CT26 xenografts	allicin 5 mg/Kg + X-ray 2Gy - 30 minutes for 21 days	↓Tumor weight ↓Tumor volume	↓ NF-κB ↓ p-NF-κB ↓ ΙΚΚβ ↓ p-ΙΚΚβ ↑ΙκΒα ↑p-ΙκΒα	[15]
C3H female mice bearing MBT-2 cells	MBT-2-B7.1 γ-irradiated 30 Gy + allicin 2 mg/day for 21 days	 ↓ Tumor frequency ↑ Cytotoxic T lymphocyte activity ↑ Macrophages and lymphocytes at tumor site 	Not reported	[173]
BALB/c nu/nu nude mice bearing SH- SY5Y xenografts	Allicin 10 mg/kg/d + cyclophosphamide 60 mg/Kg for four weeks	↓ Tumor weight ↑ Survival ↓ CD4+/CD8+ T cells	↓ VEGF	[109]
BALB/c mice bearing BEL7402 xenografts	Allicin 1 mg/m + DOX 0.2 mg/ml for 14 days.	↓ Tumor growth ↑ Apoptosis	↑ Bax ↑ FasL	[125]

Abbreviations: 5-FU (5-fluorouracile); 8-OHdG (8-Oxo-2'-deoxyguanosine); AIF (mitochondrial apoptosis-inducing factor); ALP (alkaline phosphatase); ALT (alanine aminotransferase); AST (aspartate aminotransferase); Bax (bcl-2-like protein 4); CAT (catalase); CK (creatine kinase); CK-MB (creatine kinase-MB); COX2 (cyclooxygenase 2); DOX (doxorubicin); FASL (FAS ligand); gGT (gamma glutamyl transferase); GPx (glutathione peroxidase); GSH (glutathione); Gy (gray); i.p. (intraperitoneal); IKKβ (inhibitor of nuclear factor kappa-B kinase subunit beta); IkBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha); IL-1β (Interleukin 1β); IFN-Υ (interferon Υ); LDH (lactate dehydrogenase); NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells); p-IKKβ (inhibitor of nuclear factor kappa-B kinase subunit beta); p-IKBα (phospho-nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha); p-mTOR (phospho-mechanistic target of rapamycin); p-NF-κB (phospho-nuclear factor kappa-light-chain-enhancer of activated B cells); PARP (Poly ADP ribose polymerase); rIL-2 (recombinant interleukin-2); SOD (Superoxide dismutase); TAM (tamoxifen); TMZ (temozolomide); TNF-α (tumor necrosis factor alpha); VEGF (vascular endothelial growth factor).

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