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Anticancer potential of allicin: A review

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ABSTRACT

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

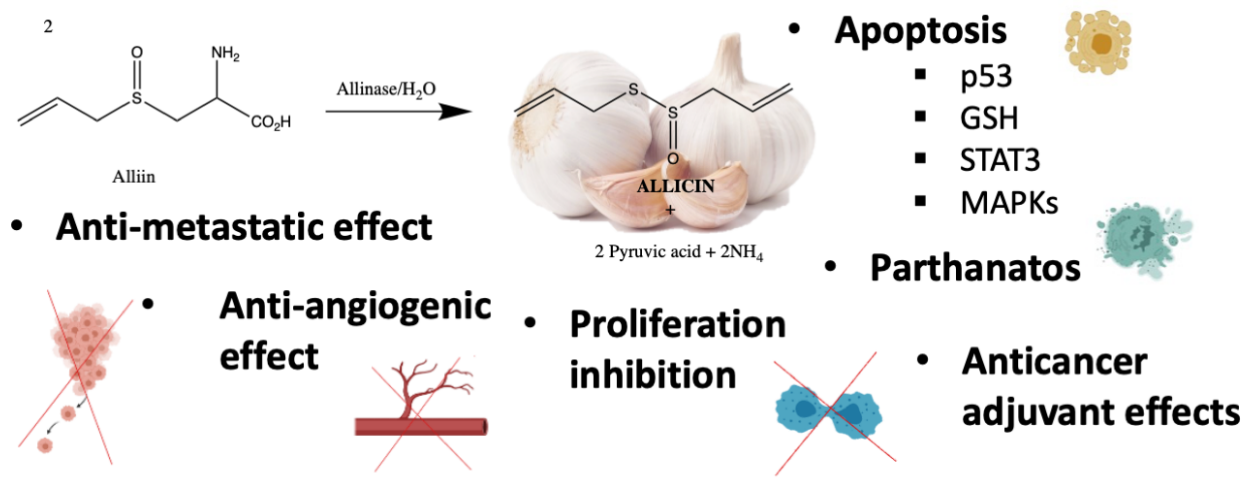
Keywords: Allicin, Anticancer Drugs, Cancer, Cytotoxicity, Pharmacokinetics, Selectivity

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; IC_{50}, half maximal inhibitory
44 concentration; IFN-β, interferon β; IκB, NF-κB inhibitory protein; IKK, IκB 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, O^6-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; PlGF, 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.

61

62 **GRAPHICAL ABSTRACT**



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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**

102 Allicin [S-(2-propenyl)-2-propene-1-sulfinothioate] is a lipid-soluble sulfenic acid thioester
103 [22]. Being very unstable, some processing and storage conditions such as concentration, pH,
104 polarity of the medium and temperature can easily lead to a spontaneous decomposition to
105 secondary organosulfur compounds as reported in figures 1, 2 and 3 [23–28].
106

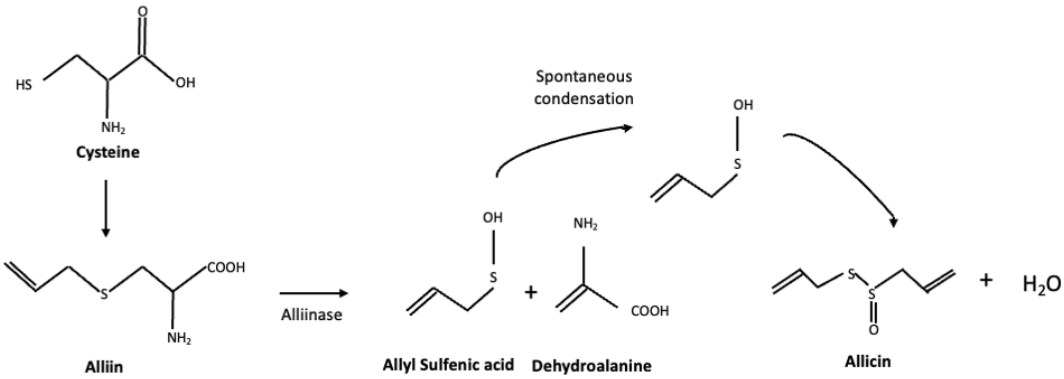

Figure 1. Enzyme-catalyzed biosynthesis of allicin
Cysteine is transformed to alliin (S-allyl-L-cysteine sulfoxide) that is hydrolyzed by the alliinase enzyme producing dehydroalanine and allyl sulfenic acid where two molecules were combined to form allicin.

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

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

129 Further studies are needed to better understand alliinase activity and consequently allicin
130 conversion 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).

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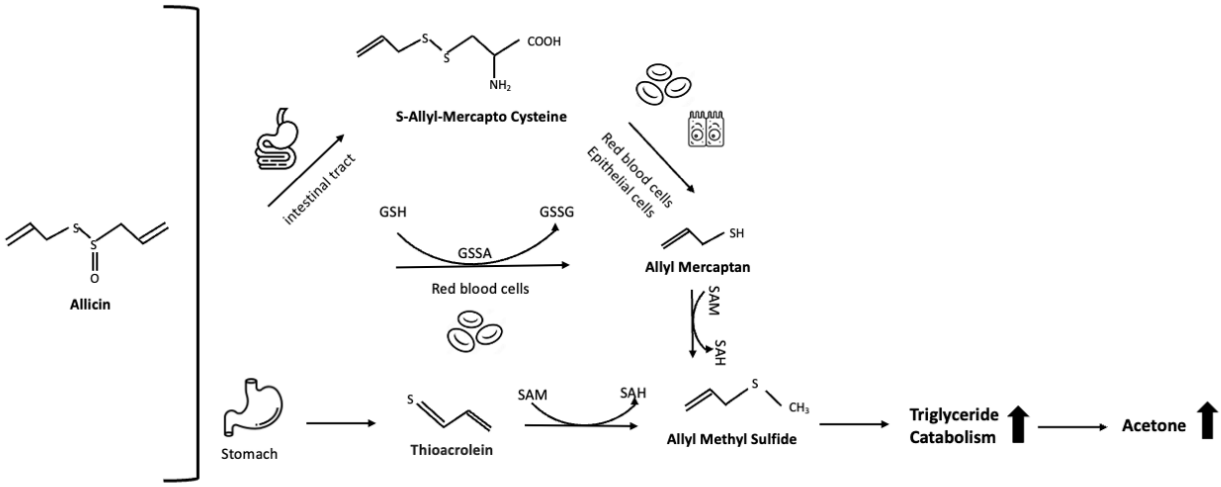

137

138 **Figure 2. Metabolic fate of allicin.** The Figure summarizes the proposed metabolic pathways of allicin. Glutathione
139 (GSH); S-allylmercaptoglutathione (GSSA); glutathione disulfide (GSSG); *S*-adenosylmethionine (SAM); S-adenosyl
140 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.

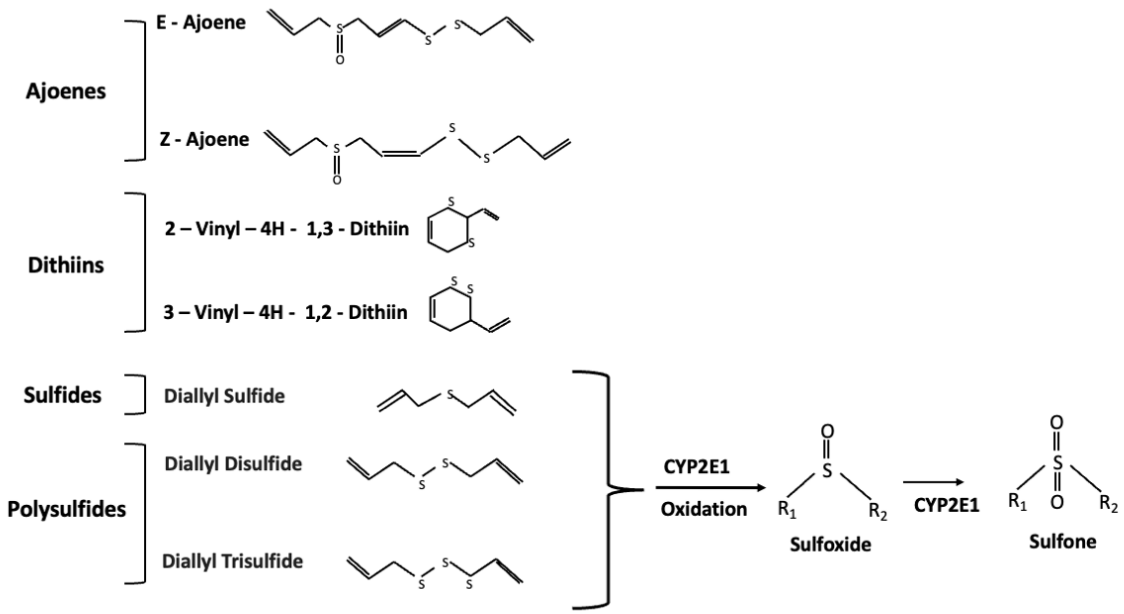

Figure 3. List and structure of some of the organosulfur compounds from allicin spontaneous decomposition and metabolism.

Some more pharmacokinetic parameters derived from an animal study showing that ^{35}S-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].

In a rat liver perfusion study, a remarkable first-pass effect of allicin was noted: 90% of allicin concentration decreased just after 3 min while 99% disappeared after 6 min. The secondary organosulfur compounds such as diallyl disulfide formed and later allyl mercaptan, ajoenes and vinyl-dithines (Figure 3) were also observed in the collected bile as well as in the liver [50–52]. The 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 22 types of garlic products, allicin bioavailability registered in healthy subjects 32h post-

183 consumption was 36–104% for enteric tablets, 26–109% for garlic powder capsules, 80–111% for
184 non-enteric tablets, 30% for roasted, 16% for boiled, 66% for acid-minced, and 19% for pickled garlic
185 foods [35].

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

193 Data from clinical studies give some more in-depth information regarding excretion. In fact,
194 by GC-MS analysis, it was shown that allyl mercaptan, AMS, allyl methyl disulfide, diallyl sulfide,
195 diallyl disulfide, and diallyl sulfone were the components, with AMS being the most abundance
196 detected in the human breath soon after the ingestion of raw garlic and commercial garlic products
197 [52], whereas AMS, allyl methyl sulfoxide and allyl methyl sulfone were the main metabolites found
198 in human milk and urine [46,47,54].

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

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

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

214 **3.1 Improvement of allicin bioavailability**

215 Allicin goes through a fast metabolism through which it is quickly inactivated. This behavior
216 clearly limits the clinical potential of this compound. Moreover, allicin is characterized by chemical
217 instability and short life. For example, it can be inactivated by heating or at a pH below 3.5 [57].
218 Therefore, enteric-coated formulations have been developed to hamper stomach disintegration of
219 many commercial garlic supplements and protect against allinase enzymes [35]. A microparticulate
220 formulation, in which alliinase and alliin are individually encapsulated inside microspheres, has been
221 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

230 (OAW42), prostate (PC3), breast (MCF7) cancer cells, and human dermal fibroblasts (HDF) and no
231 cytotoxicity has been recorded [59]. On MIA PaCa-2, the treatment with this conjugate system,
232 followed by 50 µM alliin, promoted proliferation inhibition and apoptosis. In particular, it promoted
233 the acetylation of the histone H3, to which the authors ascribe the ability to activate $p21^{Waf1/Cip1}$ and
234 the following cell-cycle arrest in the G1 phase. Apoptosis was associated with oxidative stress, as
235 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.

271 Allicin can promote: 1) indirect DNA protection (antioxidant activity and modulation of
272 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

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

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

285 Allicin, at the concentrations 1.25 mg/mL (7.70 mM), demonstrated antigenotoxic activity in
286 a mutant bacterial cell assay in which it appeared effective in decreasing methotrexate genotoxicity,
287 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
294 treated with increasing concentrations of estradiol 17-β (10-20-40 μM) yielded a concentration-
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 H_2O_2 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 µM) prevented the genotoxic effect of aflatoxin B1 (AFB1) starting at 5 µM
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 1β (IL-1β), 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-1β, and

323 chemokines, such as IL-8, is mediated by nuclear factor kappa-light-chain-enhancer of activated B
324 cells (NF-κB) [76]. However, TNF-α itself is a primary inducer for NF-κB 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-κB
327 inhibitory protein IκB. In response to a wide range of stimuli, the activation of the enzyme IκB kinase
328 (IKK) can occur, resulting in phosphorylation of IκB, which is ubiquitinated and then degraded,
329 thereby releasing NF-κB [76]. NF-κB 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-1β 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 IκB 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-κB 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

369 the converging point with the intrinsic pathway. Subsequently, BID translocates to the
370 mitochondrion, promoting the activation of Bcl-2-like protein 4 (Bax) and Bcl-2 antagonist/killer
371 (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 Ca^{2+} 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).
397 In the same cell line, another study showed that a 10-time lower concentration of allicin also
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 µg/ml (73.9 µM); 48h] [86], luminal A (MCF7; 40 µM; 24h) and triple-negative (HCC-70; 20 µM; 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
415 miR-383-5p, have been shown to play a role in cell proliferation, apoptosis, and differentiation. In

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 µg/mL (61.61 µM, 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.

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

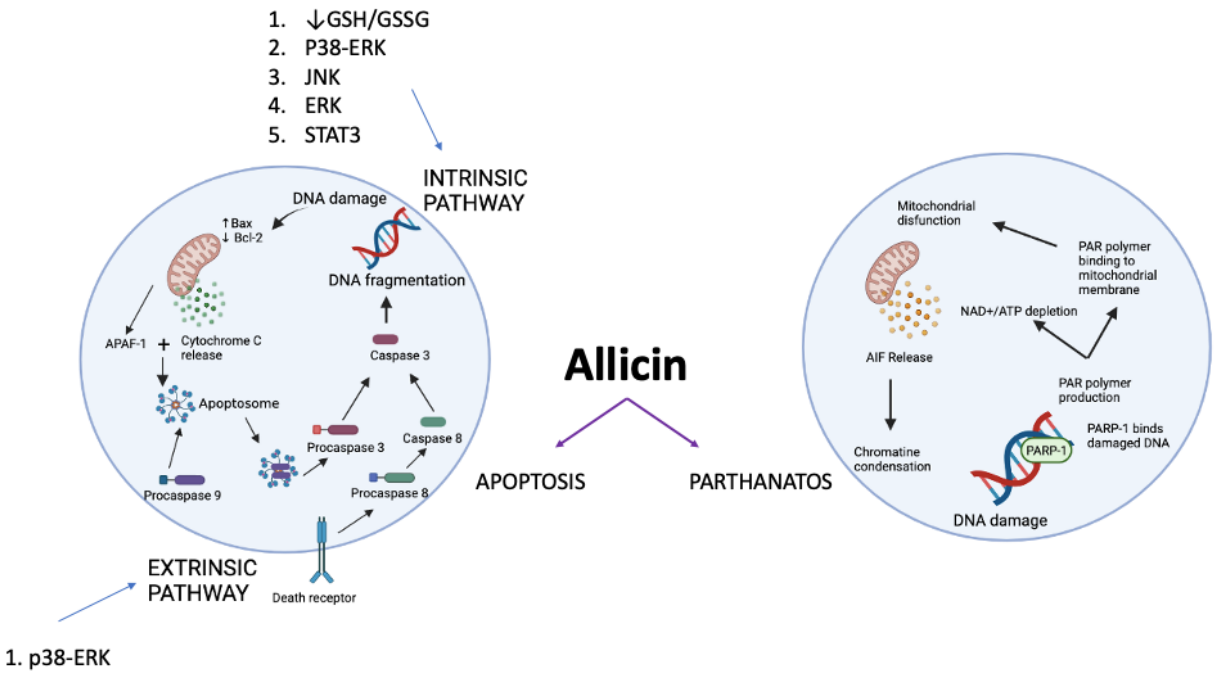

Figure 4. Schematic representation of allicin-mediated cellular death pathways. GSH (glutathione); GSSG (glutathione disulfide); ERK (extracellular signal-regulated kinase); JNK (c-Jun N-terminal kinase); STAT3 (signal transducer and activator of transcription 3); Bax (Bcl-2-like protein 4); Bcl-2 (B-cell 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

Although allicin is a reactive sulfur compound that quickly oxidizes -SH groups, such as those in GSH or those present in any cysteine residue of different proteins, it also acts as an antioxidant thanks to a sort of rebound effect. Indeed, oxidative stress can be detrimental for cells, but it also activates an antioxidant response mediated by oxidative stress protection responses [1].

Certainly, as a consequence of oxidative stress in general, and GSH drop in particular, as a defense mechanism, cells promote the activation of nuclear factor-erythroid factor 2-related factor 2 (Nrf2) and its translocation into the nucleus. Nrf-2 is a critical transcription factor that modulates more than 1000 genes in normal and stressed circumstances. Nrf2 endorses the synthesis of several proteins having different roles, such as an antioxidant, detoxifying, anti-inflammatory [95]. It is not 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 µg/mL (61.61 µM)
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 GSH
481 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**

498 The stress-activated protein kinases, or MAPKs family, represent one of the fundamental
499 intracellular signal transduction systems in the monitoring and surveillance of cell survival,
500 proliferation, differentiation, and cell death [102–105]. MAPKs include three main signaling
501 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 µg/mL (154.04 µM, 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 hypoxia-

526 inducible factor (HIF) 1α and HIF2α 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.

528 **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**

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

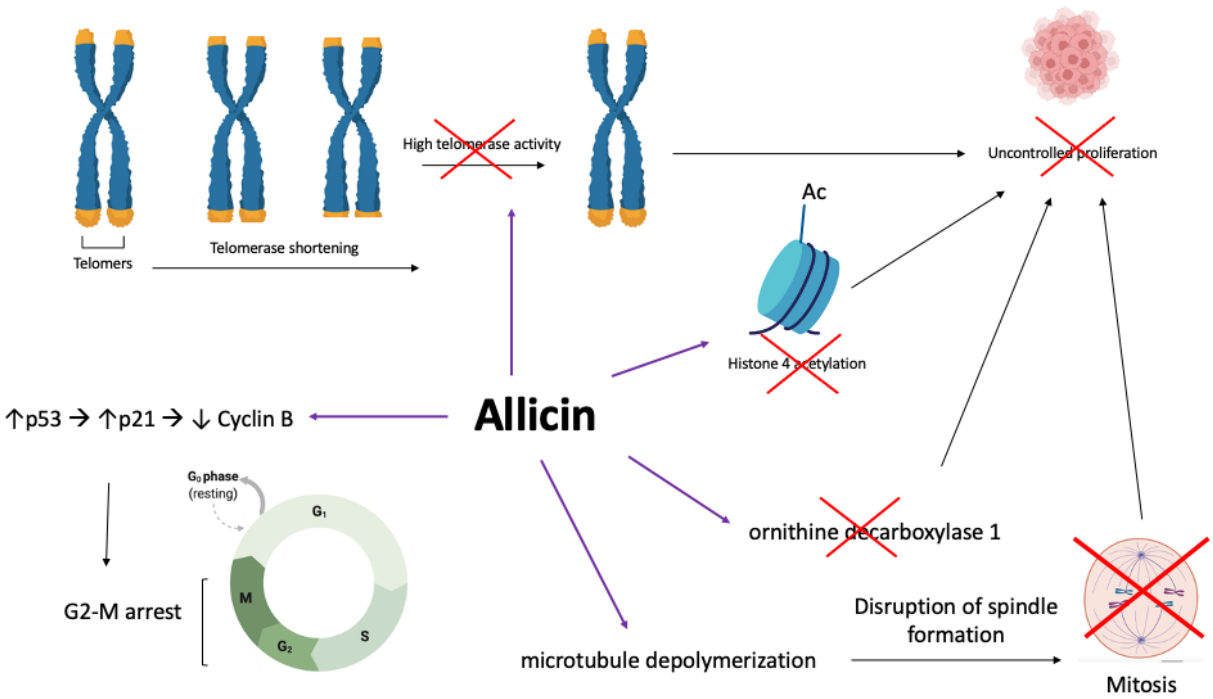

Figure 5. Schematic representation of allicin-mediated antiproliferative pathways. Created with BioRender.com.

At both low [leukemia HL-60 cells (5 µM, 20h)] [2] and high concentrations [SGC-7901 (616,14 µM, 24h [6], Caco-2 (500 µM, 24h) [90], and A549 (246.34 µM, 48h] [109]], allicin promoted a cell-cycle arrest at G2/M phase (table 1). In the case of SGC-7901, the antiproliferative effect could be due to the inhibition of telomerase activity produced by allicin at the same conditions [6]. Telomeres are non-coding, tandemly repeated nucleotide sequences found at the end of eukaryotic chromosomes. They stabilize DNA, preventing chromosomes from wrapping around themselves or recombining at their ends. Under physiological conditions, somatic cells present a very low or absent telomerase activity, which does not allow the synthesis of new telomeres. Consequently, at each replication cycle, telomere sequences shorten to a critical length, beyond which mitosis stops and cells enter a phase known as "senescence". Senescent cells remain metabolically active, but their gene expression is altered and division cycles slow down [111]. However, senescence, despite being a physiological process, represents a way for tumor cells to acquire resistance towards chemotherapeutics and can cause relapses and favor formation of metastasis [112]. For example, double-negative MCF7 and triple-negative HHC-70 breast carcinoma cells develop resistance to

561 doxorubicin (DOX) by going through senescence. Allicin 45 µM (MCF7) and 20 µM (HHC-70) showed
562 a senolytic and antiproliferative effect on both these DOX-resistant senescent cells [87]. Thus, we
563 can speculate that although allicin inhibits telomerase activity, it does not activate senescence,
564 which for tumor cells represents a chemoresistant marker, but only blocks cell proliferation ending
565 in cell death.

566 On non-resistant MCF7, instead of the senolytic effect, allicin promoted the accumulation of
567 cells in the G1 (20 µM) or the G1 and G2/M phases (30 µM) 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 µM 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].

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

623 Tumors cannot exceed 1-2 mm in size unless they are vascularized. That is, in fact, the
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 PlGF (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 µM 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]. HIF1α 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 µM, 48h) showed a protective role by opposing to the increase in overexpressed HIF1α and
651 VEGF, confirming antiangiogenic potential [11] (Figure 6).

652 Allicin 10 µM inhibited both angiogenesis and lymphangiogenesis. *In vitro* and *in vivo*, it
653 blocked VEGF-C-mediated tube formation and reduced the infiltration of ECs and leukocytes. In
654 addition, *in vitro*, it blocked lymphatic HMVEC-dLy cell migration [123]. During the epithelial-
655 mesenchymal transition (EMT), epithelial cells lose their phenotype and acquire the characteristics
656 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).

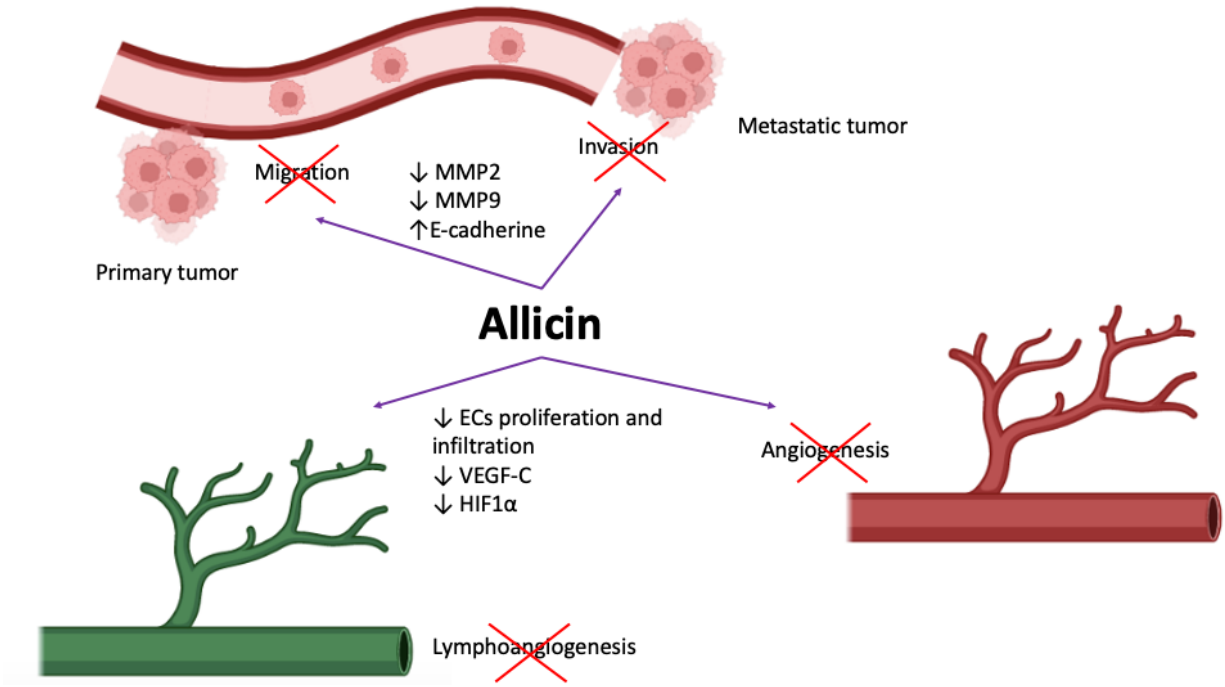

Figure 6. Allicin-mediated anti-angiogenic and -metastatic pathways. Created with BioRender.com.

5. In vivo studies

A few studies confirmed allicin antitumor potential *in vivo*. Allicin can counteract tumor growth *in vivo* in different tumor mice models, such as cholangiocarcinoma [5], colon cancer [13], lymphoma (table 2) [4]. As for the *in vitro* experiments, the actual mechanism of action has not been 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 µg +
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 dose-
685 dependent response [3]. To support this assumption, a different study showed that in CH3 mice
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].

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

695 routes (oral, intraperitoneal and intravenous). Thus, Ehrlich-2 ascites carcinoma or sarcoma 180
696 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
709 allicin + RD and allicin or RD alone. However, in contrast to MD or RD alone or in combination with
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.

719 On the whole, these results disclosed the antitumor efficacy of allicin *in vivo* in fighting
720 different neoplasms. Only few studies have monitored the safety of allicin use, showing a very
721 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**

732 The first example is the synergistic activity recorded with 5-fluorouracil (5-FU) on
733 hepatocellular [128], colorectal, and lung [129] cancer cells. Allicin increased the growth inhibition
734 potential of 5-FU and amplified apoptosis both *in vitro* (table 3) (BEL-7402, SK-Hep-1, DLD-1, and
735 SK-MES-1) and *in vivo (*SK-Hep-1 xenografted mice) (table 4), indicating synergism [128].
736 Engaging the immune system in the fight against cancer represents one of the most successful
737 strategies conceived so far. Many different types of immunotherapies have been developed, and
738 the impressive positive outcomes demonstrate that the rise of an immune response is very
739 favorable not only to eradicate tumors but also to avoid chemoresistance and relapses [130]. IL-2 is
740 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-γ 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.

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

757 Allicin has been tested *in vitro* and *in vivo* with another natural compound having antitumor
758 activity, artesunate. The association of these two molecules increased the cytotoxic effect of each
759 single agent alone on different osteosarcoma cell lines (MG-63, U20S, 143-B, SaOS-2, and HOS)
760 (table 3). On MG-63 and U20S, allicin + artesunate ameliorated the proapoptotic activity and limited
761 the ability of tumor cells to migrate. *In vivo* experiments confirmed the interesting effects of this
762 combination, and allicin + artesunate suppressed tumor growth to a higher degree than single
763 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].

788 Tamoxifen (TAM) is another powerful but toxic anticancer drug widely used to treat hormone-
789 dependent breast cancer. One of the many adverse effects induced by TAM is liver injury. Allicin
790 increased TAM's antitumor potential *in vitro* on Ehrlich ascites carcinoma (EAC) cells (table 3), and
791 *in vivo* in a mouse model of EAC (table 4). In the experimental animals, allicin also counteracted the
792 liver injury induced by TAM preventing oxidative stress, lipid peroxidation, and hepatic
793 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-κB 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 IκBα phosphorylation [15].

804 The tumor suppressor miR-486 is often downregulated in different tumors, such as
805 glioblastoma, and can cause chemoresistance. miR-486 directly binds O^6-methylguanine-DNA
806 methyltransferase (MGMT), an enzyme that actively promotes DNA repair. Basically, MGMT
807 protects cells from alkylating agents. If these cells are tumor ones and the alkylating agent a
808 chemotherapy one, it is easy to understand that its overexpression promotes the rise of resistance,
809 and its antagonization represents an antitumor strategy [143], [144]. Glioblastoma cells usually
810 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
822 tumor types [135,143,147], such as myeloma [148–150]. The effect of the combinatorial treatment
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].

829 5-FU is approved for the treatment of gastric carcinoma, but chemoresistance limits its
830 application. The anticancer effects of allicin, 5-FU, and allicin/5-FU on the 5-FU resistant gastric
831 MKN-45 cancer cells were evaluated by MTT assay and DAPI staining. The expression of the P-
832 glycoprotein (P-gp) and CD44 proteins was also determined using immunocytochemistry. The
833 combination of allicin with 5-FU significantly increased apoptosis and decreased the expression of
834 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 µg/mL – 30.08 µM) 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**

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

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

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

881 respectively. Besides, the third one did not respond to allicin at all. In this study, as a reference,
882 human mammary MCF7, endometrial Ishikawa, and colon HT-29 cancer cells showed to be more
883 sensitive than the *ex vivo* fibroblasts since the IC_{50} were all comprised between 10 and 25 µM. A
884 similar outcome happened testing allicin on lymphoma L5178Y and normal spleen cells: IC_{50} on
885 tumor cells was exactly 2.46 times smaller than that recorded for spleen cells (443.62 µM *versus*
886 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 µM. 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.

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

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

934 **8. Conclusions**

935 Cancer is a leading cause of death globally [159]. Consequently, finding novel therapeutic
936 strategies and less toxic natural-based compounds are mandatory for the treatment of patients.
937 Allicin is a natural product widely consumed in most cultures being one of the active garlic
938 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

952 between phytochemicals and traditional anticancer drugs could be clinically advantageous, the type
953 of interaction needs to be carefully explored in animal models and clinical trials to exclude
954 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 clinicaltrials.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].

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

984 Regarding the epidemiological evidence, the major studies show protective effects of garlic
985 against gastro-intestinal cancer. The most recent meta-analysis was conducted on a total of 8,621
986 cases and 14,889 controls: garlic intake was associated with reduced risk of gastric cancer [168]. The
987 findings on colorectal cancer are controversial. Based on a recent meta-analysis including 11 studies
988 and involving 12,558 cases, garlic intake could reduce the risk of colorectal cancer [169], but this
989 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 reported	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]
HT-29	500-1000 µM, 48 h	Apoptosis, proliferation inhibition	G2/M cell-cycle arrest ↑ Phospho-Histone3	[90]
	10-40 µM	Cytotoxicity	Not reported	[9]
	37.5-70 µM, 24h	Cytotoxicity	↓ GSH ↑ ROS	[91]
	10 µg/ml, 24 - 72h	Cytotoxicity	Not reported	[14]
Caco2	10 µg/ml, 24 - 72 h	Cytotoxicity	Not reported	[14]
	500-1000 µM, 48 h	Apoptosis, proliferation inhibition	↓ ΔΨm G2/M cell-cycle arrest ↓ 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]

			↑ AIF ↑ EndoG ↑ Htra2/omi ↑ Caspase 3 ↑ Caspase 8 ↑ Caspase 9 ↓ ΔΨm	
Hep3B p53 mutation	35 µM, 24h	Apoptosis	↑ ROS ↑ Bax ↓ Bcl-2 ↑ AIF ↑ EndoG ↑ Htra2/omi ↑ Caspase 3 ↑ Caspase 8 ↑ Caspase 9 ↓ ΔΨm	[100]
PC-3	77,92 µM, 24h	Cyotoxicity	Not reported	[7]
SK-N-FI	IC50 72 h: 18.6 µM	citotoxicity, apoptosis	↑ PARP Ornithine Decarboxylase Inhibition	[116]
SK-N-AS	IC50 72 h: 19,48 µM	citotoxicity, apoptosis	↑ PARP Ornithine Decarboxylase Inhibition	[116]
SK-N-Be(2)c	IC50 72 h: 10,27 µM	citotoxicity, apoptosis	↑ PARP Ornithine Decarboxylase Inhibition	[116]
Kelly	IC50 72 h: 9,21 µM	citotoxicity, apoptosis	↑ PARP Ornithine Decarboxylase Inhibition	[116]
U87MG	90 µM, 24h	Apoptosis	↑ Bax/Bcl-2 ↑ p38 ↑ pERK	[96]
HMCV-transfected U87MG	60 µg/mL, 24-168h	Cytotoxicity	↑ p53 ↓IL-6 ↓IFN-β	[101]
Tca-8113	12.5 - 50 µg/mL , 24-48 h	Apoptosis	↑ Bax ↓ Bcl-2 ↑ Caspase 3	[86]
SCC-25	12.5 - 50 µg/mL 24-48 h	Apoptosis	↑ Bax ↓ Bcl-2 ↑ Caspase 3	[86]
SGC-7901	15 - 120 µg/mL, 24-72h	Apoptosis	↑ Cytochrome c release ↑ Caspase 3	[84]

			↑ 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 µM	Apoptosis	↑ Apoptotic bodies ↑ 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]
A549	36 µM, 24 h	Cyotoxicity	not reported	[7]
	30 µg/mL, 72h		Not reported	[10]
	37.5 – 188 µM, 24h		↓ 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	↑ Bax ↑ AIF ↑ 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]
HCT116	25 µM, 24 h	Apoptosis	↓ pSTAT3 ↓MCL-1, ↓Bcl-2 ↓Bcl-xL	[13]
	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 (myeloid 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-3-kinase); 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 domain-containing 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	↓ 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.	↓ **T**umor 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 µg/mL + temozolomide (TMZ) 50 µg/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	↑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 ↓ IKKβ ↓ p-IKKβ ↑IκBα ↑p-IκBα	[15]
CD44+ A375	Allicin 5 µg/mL + ATRA 37.43 µM for 48h	↑ Cytotoxicity	Cell-cycle arrest in S phase ↑MMP-9 ↑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	↑ 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	↑ Cytotoxicity ↑ 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); IκBα (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-κB (nuclear factor kappa-light-chain-enhancer of activated B cells);); p-AKT (phospho-protein kinase B); p-IKKβ (inhibitor of nuclear factor kappa-B kinase subunit beta); p-IκBα (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); 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	↑INF-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 ↓ IKKβ ↓ p-IKKβ ↑IκBα ↑p-IκBα	[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); IκBα (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-IκBα (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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