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In Vivo Antitumor Activity of Allicin in a Pediatric Neuroblastoma Patient-derived Xenograft (PDX) Mouse Model

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Abstract

Background/Aim: Allicin is a small-molecule natural product found in garlic (Allium sativum). We previously showed that allicin inhibits ornithine decarboxylase (ODC) *in vitro* and induces apoptotic cell death in pediatric neuroblastoma (NB) cancer cell cultures. However, its potency as an anticancer agent *in vivo* has not been sufficiently explored.

Materials and Methods: In this study, we used cell proliferation assays, immunoblotting techniques, and light microscopy to study NB tumor cell cultures and human primary neonatal skin fibroblast control cells as well as a *MYCN*-amplified NB patient-derived xenograft (PDX) mouse tumor model to study the efficacy of allicin *in vivo*.

Results: Allicin strongly inhibits NB tumor cell proliferation in a dose-dependent manner while non-cancerous human primary neonatal skin fibroblast control cells were largely unaffected. Importantly, two intra-tumoral injections of allicin over a two-week trial period significantly reduced the NB tumor burden in mice compared to controls (N=4-9 mice/group). Excised tumor tissues revealed that allicin treatment increased the cyclin-dependent kinase inhibitor $p27^{Kip1}$ protein levels, suggesting that *in vivo*, allicin increases $p27^{Kip1}$-mediated G_1/S cell cycle arrest.

Conclusion: Our findings warrant further preclinical development of allicin as a potential anticancer agent, especially for those types of cancers that are treatable by intra-tumoral injections, including neuroblastoma, glioblastoma, and medulloblastoma.

Keywords: Allicin, childhood cancer, *in vivo* antitumor activity, intra-tumoral injection, natural products, neuroblastoma, patient-derived xenograft (PDX).

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Introduction

When tissues of garlic (*Allium sativum*) are damaged they release the natural product allicin, the pungent-smelling volatile organic sulfur compound (VOSC), that is the typical odor of fresh garlic (1, 2) (Figure 1A and B). Allicin reacts reversibly with cellular thiols (R-SH) by making a disulfide bridge with an allyl group attached, *i.e.*, R-SH becomes R-S-S-CH_2-CH=CH_2. The low molecular weight thiol glutathione and proteins containing cysteine are typical cellular targets for allicin and the disulfide bridge in the thioallylated products can be reduced back to thiols, thus making allicin effects reversible at sublethal levels. When cellular proteins are affected in this way their activity can become altered, and this makes allicin a dose-dependent biocide (1). The *S*-thioallylation reaction is central to allicin's biological reactivity and, significantly, has also been shown to be performed by other compounds like allyl polysulfanes, found in aged garlic products and garlic oils where no allicin is present (3).

MYCN gene amplification is often associated with high-risk neuroblastoma (NB), a fatal pediatric cancer, where relapsed tumors are virtually untreatable (4-6). The transcription factor MYCN directly activates the gene ornithine decarboxylase 1 (*ODC1*) (7, 8) and the ODC protein is a rate-limiting enzyme in polyamine biosynthesis (9-11). We have been interested in the role of ODC and polyamines in NB for many years (12-20). High polyamines (putrescine, spermidine, spermine) trigger cell proliferation in NB as well as MYC-driven cancers (21-24). Difluoromethylornithine (DFMO, also known as Eflornithine, Ornidyl, and Iwilfin) is currently the only FDA-approved ODC inhibitor and has various challenges in the clinic, including high dosing requirements and rapid renal clearance. Because ODC has multiple cysteine residues, we tested whether human ODC was inhibited by allicin *in vitro*. We found allicin to be approximately 23,000 times more effective than DFMO, an astounding result that prompted us to also test the activity of allicin in NB cell cultures. Allicin indeed inhibits endogenous ODC activity, reduces polyamines, and induces apoptotic death in NB cell cultures (25).

The primary purpose of this study was to test the therapeutic activity *in vivo*, by injecting allicin directly into NB patient-derived xenograft (PDX) tumors.

Materials and Methods

Chemicals, reagents, and antibodies. Allicin was synthesized as previously described (26) and suspended in a 48 mM stock solution in water. Rabbit polyclonal antibody against PARP (#9542) and rabbit monoclonal antibody against $p27^{Kip1}$ (#3686) was obtained from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibody against GAPDH (SC-47778) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Goat anti-rabbit secondary antibody conjugated to IRDye®680 RD (926-68071) and goat anti-mouse secondary antibody conjugated to IRDye®800CW (926-32210) were obtained from LI-COR Biosciences (Lincoln, NE, USA). Protein assay dye reagent (#5000006) was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

Cell culture. Human NB cells lines were purchased between 2014 and 2016 from certified suppliers. Cells were cryopreserved in liquid nitrogen and used for cell culture experiments as previously described (25). SK-N-FI were from the Childhood Cancer Repository/Children's Oncology Group Resource Laboratory, SK-N-AS (CRL-2137), and SK-N-BE from the ATCC (Manassas, VA, USA), and Kelly (CB_92110411) from Sigma-Aldrich (St. Louis, MO, USA). The SK-N-BE clone used was SK-N-Be(2)c (ATCC: CRL-2268). The MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza, Basel, Switzerland) was used to monitor cells lines yearly for mycoplasma contamination. NB cells were cultured in RPMI 1640 (VWR, Radnor, PA, USA) containing 10% heat-inactivated fetal bovine serum (Invitrogen, Waltham, MA, USA), Penicillin (100 IU/ml) and Streptomycin (100 μg/ml) (30-002-CI, Corning, Corning, NY, USA). The human primary neonatal skin fibroblasts were grown in DMEM medium (Gibco, Waltham, MA, USA) containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA), penicillin (100 IU/ml), and

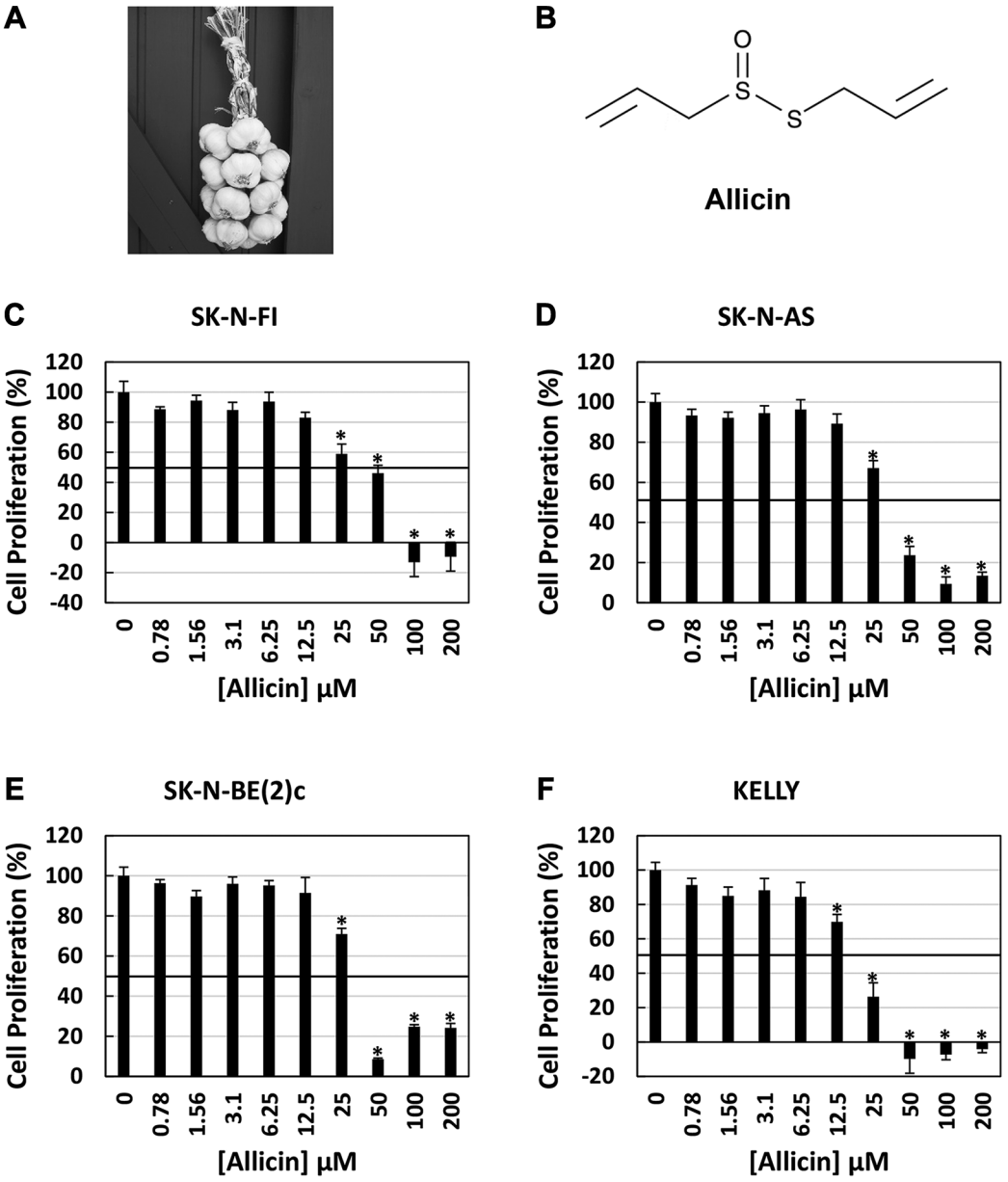

Figure 1. *Dose-dependent effect of allicin in neuroblastoma cell cultures. The chemical allicin (B) is a natural product extracted from garlic (A). Increasing concentrations of allicin inhibited NB cell proliferation in a dose dependent manner (C-F) with the greatest effect in SK-N-FI (C) and KELLY (F) cells. Data represents the mean±the standard deviation (S.D.) of a single experiment done in quadruplicate (N=4). *Statistically significant decrease in cell proliferation as compared to control (p=0.0001 or less).*

streptomycin (100 μg/ml), non-essential amino acids (0.1 mM) and sodium pyruvate (1 mM). NB cells were grown in a humidified atmosphere at 37˚C with 5% CO_2. Cells were 80-90% confluent when counted for use in experiments.

Immunoblotting. Tumor and cell protein lysates were harvested, with equal amounts of protein resolved by Western blotting as previously described (27). PARP, $p27^{Kip1}$, and GAPDH levels were detected by incubating

blots with primary antibodies diluted 1:1,000 in 5% BSA in Tris-buffered saline (TBS) with 0.1% Tween-20 overnight at 4˚C. Blots were washed with TBS containing 0.1% Tween-20 three times for 5 min each. Secondary antibodies containing fluorophores were diluted 1:1000 in 5% non-fat dry milk in TBS containing 0.1% Tween-20 and incubated at room temperature for 1 h. Blots were again washed with TBS containing 0.1% Tween-20 three times for 5 min each. Images of the blots were captured using an Odyssey Clx (LI-COR Biosciences, Lincoln, NE, USA) Western blot scanner. Image Studio Lite (LI-COR Biosciences) software was used to quantitate the relative $p27^{Kip1}$ signal.

Cell proliferation assay. The colorimetric sulforhodamine B (SRB) assay (28, 29) was used to measure the cellular proliferation of actively growing NB cells following treatment with increasing concentrations of allicin as previously described (25, 30). For NB cells treated with allicin, a single experiment was performed in quadruplicate for each condition. For neonatal skin fibroblast cells treated with allicin, three independent experiments were performed (N=16).

Microscopy. Light micrographs of the human primary neonatal skin fibroblasts treated with allicin were captured at 10X using a camera attached to a Leica DMi1 microscope.

Animal ethics. All experimental animal procedures were approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (IACUC, Protocol #PROTO202100058). MSU is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-certified institution.

In vivo tumor studies. The effects of allicin on *in vivo* NB tumor growth was performed using the NB patient-derived xenograft (PDX) line COG-N-623 (Children's Oncology Group Childhood Cancer Repository). This PDX line was derived from a *MYCN*-amplified NB tumor established at time of disease progression after chemotherapy (31). Six- to eight-week-old athymic nude mice were injected with 1.5×10^7 COG-N-623 tumor cells and tumor volume was measured and calculated as previously described (27). Mice containing palpable tumors (~100 mm^3) were randomized into 4 treatment groups: 1. Phosphate Buffered Saline (PBS) control (n=7); 2. 0.05 mg allicin in PBS (n=8); 3. 0.2 mg allicin in PBS (n=9); 4. 0.5/0.2mg allicin (n=4). For each group, the treatment was administered once weekly in 100 microliters *via* intra-tumor injection, distributing the volume across multiple quadrants of the tumor. After the first treatment, it was noted that the 0.5 mg allicin treated tumors exhibited excessive bleeding and their dose was cut to 0.2 mg allicin for subsequent treatments. Tumor volumes were measured 3 times per week. Tumors were treated for 2 weeks. At this point control tumors had to be euthanized since they had grown to approximately 2,500 mm^3 in size. Mice were euthanized with CO_2 using a Euthanex Prodigy system, which is compliant with the American Veterinary Medical Association (AVMA) guidelines. The tumors were removed, weighed, snap frozen with liquid nitrogen, and stored at –80˚C.

Statistical analyses. An unpaired Student's *t*-test assuming the null hypothesis was used to determine the statistical significance of allicin treatment on neuroblastoma cell proliferation, tumor volume and relative tumor $p27^{Kip1}$ expression. Unless otherwise noted in the figure legend, for all comparisons, $p<0.05$ was considered statistically significant.

Results

Allicin inhibits tumor growth of neuroblastoma cells. Allicin is a natural product produced by the garlic plant (Figure 1A and B). We previously showed that chemically synthesized allicin with high purity induces apoptotic cell death in NB tumor cells (25). Herein we confirm that allicin inhibits the proliferation of the four NB tumor cell lines SK-N-FI, SK-N-AS, SK-N-BE(2)c, and KELLY, in a dose-dependent fashion after 24 h of treatment (Figure 1C-F). The approximate concentration of allicin required to

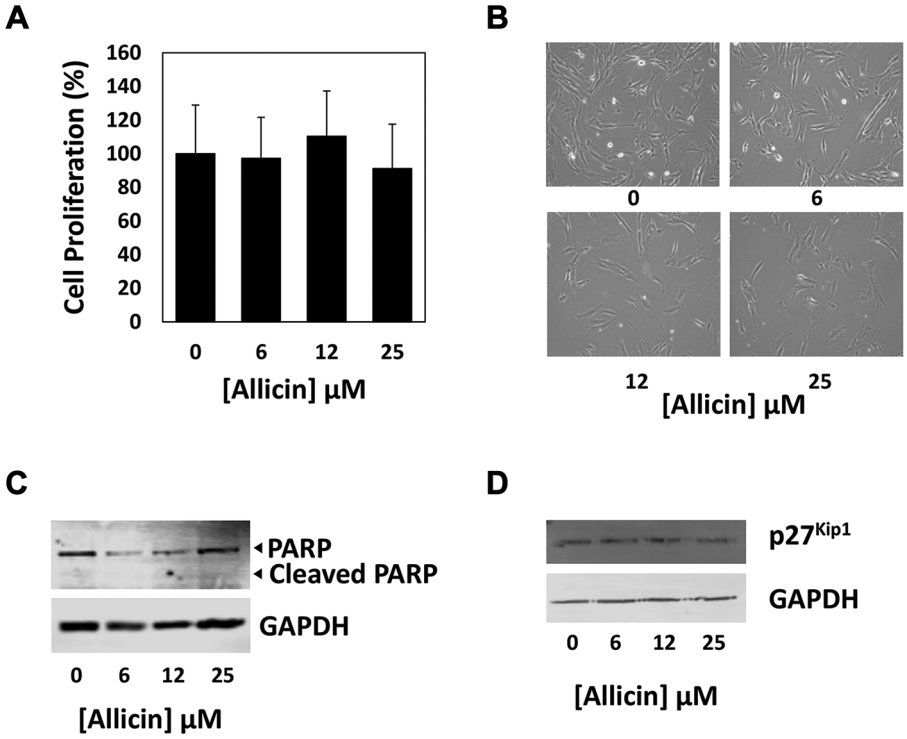

Figure 2. *Effect of allicin on human primary control cells. Human primary neonatal skin fibroblasts treated with allicin (0, 6, 12, 25 µM) for 24 h. Allicin did not affect human primary neonatal skin fibroblast control cell proliferation after 24 h of treatment (A). Light micrographs show that allicin does not induce the detachment of adherent cells and the formation of rounded cell phenotypes, a hallmark of apoptosis (B). Allicin did not induce apoptosis in human primary neonatal skin fibroblast control cells, as indicated by the lack of cleaved PARP using the immunoblotting detection method (C). Likewise, there were no changes in the cell cycle marker p27^{Kip1} (C). Data represents the three independent experiments (N=16)±the standard deviation (B). Western blot images are representative of 2 (p27^{Kip1}) or 3 (PARP) independent experiments.*

inhibit 50% cell growth was estimated in the range of 19-50 µM depending on the cell line and was most effective against the KELLY NB tumor cell line (Figure 1F).

Allicin does not induce cell death in human primary neonatal skin fibroblasts. To determine if allicin also inhibits non-cancerous human cells, we treated human primary neonatal skin fibroblast control cells with allicin in a dose-dependent manner (0, 6, 12, 25 µM) for 24 h. As shown in Figure 2A, allicin did not significantly affect cell proliferation of control cells. To determine if allicin induces apoptotic cell death, we examined these normal human control cells by light microscopy and confirmed that allicin does not induce cell detachment and the formation of rounded cell phenotypes, a hallmark of apoptosis (Figure 2B). Furthermore, the detection of PARP protein expression by immunodetection confirmed that apoptosis is not induced as judged by the lack of cleaved PARP product (Figure 2C). In addition, allicin also did not induce p27^{Kip1}, a common marker of cytostasis and G_1/S-linked cell cycle arrest (Figure 2D). In stark contrast, we previously published that allicin induces strong apoptotic cell death in four NB tumor cell lines (SK-N-FI, SK-N-AS, SK-N-BE(2)c, and KELLY) as judged by PARP cleavage and rounded cell detachment (25). These results suggest that allicin only kills NB cancer cells by means of apoptosis but not normal human primary cells, which is an important observation indicating that future treatments with allicin may not lead to adverse side-effects in normal tissues.

Allicin inhibits PDX neuroblastoma tumor growth in vivo. To investigate if allicin inhibits tumor growth *in vivo*, we performed studies in mice using a PDX of human NB. All PDX tumors were treated with 0.05 mg, 0.2 mg, and 0.5/0.2 mg allicin per intra-tumoral injection or PBS (control) when tumors were palpable (day 0) and again on day 7 of the trial. On day 14, tumors in the control group and lowest allicin dose treatment group reached ≥2,500 mm^3 in size, thus requiring euthanasia of these mice per internal IACUC guidelines. As shown in Figure 3, the tumor volumes of mice that received two treatments of allicin at 0.2 mg/injection were significantly smaller than tumors of control mice, as early as 7 days after the first injection as well as on day 14 of the study ($p<0.05$). Injecting tumors with 0.5 mg (day 0) and 0.2 mg (day 7) allicin further suppressed tumor growth ($p<0.05$), showing that allicin is an effective antitumor agent and able to inhibit NB PDX in mice.

Allicin induces $p27^{Kip1}$ protein expression in excised neuroblastoma PDX tumor tissues. Subsequent to our mouse study (Figure 3), all tumors were excised at the conclusion of the mouse study, and tumor tissues were further analyzed by western blot and immunodetection. We found that the cyclin-dependent kinase inhibitor $p27^{Kip1}$ significantly increased in allicin-treated tumors ($p<0.05$), indicating that *in vivo*, allicin induces $p27^{Kip1}$-mediated G_1/S cell cycle arrest (Figure 4). Additional protein markers including the tumor suppressor protein p53 and PARP did not change in response to allicin treatments (not shown), suggesting that unlike what we observed in cell cultures (25), allicin appears to suppress tumor growth *in vivo* by stalling cell cycle progression.

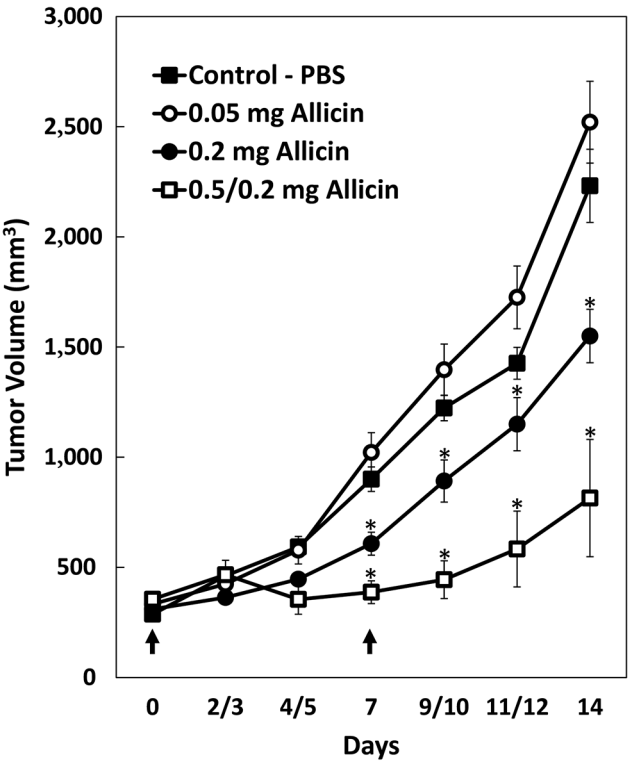

Figure 3. *Antitumor efficacy of allicin in vivo. Mice harboring NB patient-derived xenograft (PDX) tumors were treated by intra-tumoral injections with 0.05, 0.2 and 0.5/0.2 mg allicin per injection when tumors were palpable (100 mm^3, day 0) and seven days later (day 7). Tumors treated with allicin exhibited a dose-dependent decrease in tumor volume. Tumors treated with 0.2 mg (N=9, green line) and 0.5/0.2 mg (N=4, yellow line) allicin were statistically significantly smaller than control tumors (N=7, blue line) starting at day 7 and continued throughout the study until day 14. Treatment with 0.05 mg allicin (N=8, red line) had no effect on tumor volume. The data represents the mean tumor volume±standard error (S.E.) at each time point. *Denotes a statistically significant decrease as compared to control (p<0.05). Black arrows denote the days of intra-tumoral allicin injections.*

Discussion

In our previous studies of the human proteome, the most prevalent cellular targets of allicin were glycolytic enzymes and cytoskeletal proteins, many of which are critical in cancer development (32). Allicin has a broad-spectrum activity and has been shown to directly inhibit a range of SH-containing enzymes (33). In addition, our own studies revealed that allicin targets ODC (25). Importantly, allicin can regulate SHP-1/STAT3, Nrf2, ROS, ERK and p38 MAPK-associated signaling cascades, all of which are key regulators of tumorigenesis (34-39). A number of synergisms have also been reported, for example, the combination of allicin with all-trans retinoic acid (ATRA), cyclophosphamide, and artesunate (40-42). Furthermore, many tumor types are associated with abnormally high GSH levels which protects the tumor cells

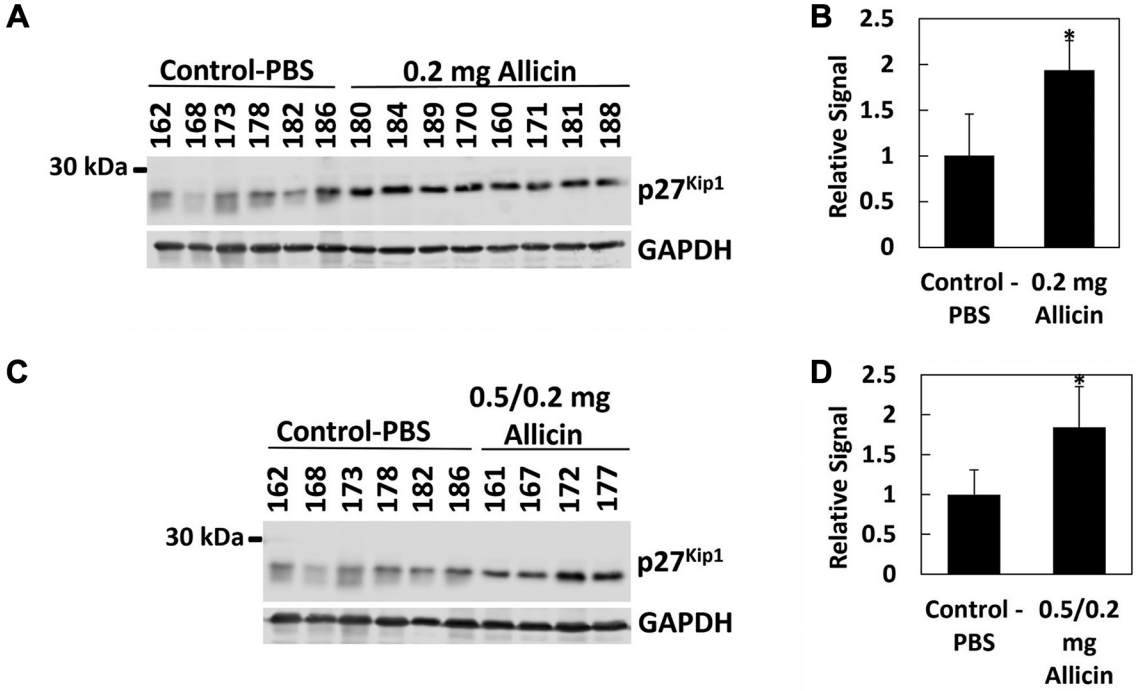

Figure 4. *Allicin treatment increases cyclin-dependent kinase inhibitor $p27^{Kip1}$ protein in NB tumors. Excised tumors were analyzed for $p27^{Kip1}$ protein levels by immunoblotting (A, C). Each number represents an individual mouse. GAPDH was used as a loading control. $p27^{Kip1}$/GAPDH signal ratios were determined using Image Studio Lite (Ver 5.2) and normalized to control (B, D). Bar graphs represent the mean relative signal±S.D. Tumors treated with 0.2 mg (A, B) and 0.5/0.2 mg (C, D) allicin had increased amounts of $p27^{Kip1}$ as compared to control. *Denotes a statistically significant increase as compared to control ($p<0.05$).*

against some therapeutic agents (43, 44). Because GSH is a primary target for allicin, it depletes the cellular GSH pool and that is likely to further help restrict tumor cells. Interestingly, it was recently shown that enhanced glutathione biosynthesis might be a selective metabolic adaptation required for initiation of *MYCN*-driven NB, suggesting that glutathione-targeted drugs might be used as a potential preventative strategy, or as an adjuvant to existing chemotherapies in established disease (45).

While several studies have shown the cytotoxic potency of allicin in cultured cancer cell lines and the induction of apoptosis (34-42, 46), fewer studies have examined its effect on normal, non-cancerous human primary cells. We here show that, at the doses employed, allicin only moderately reduced the growth of human primary neonatal skin fibroblasts, without inducing apoptotic cell death (Figure 2). Therefore, allicin is cytotoxic against NB cancer cells only and not normal cells at identical doses, suggesting that future drug treatments with allicin will likely not induce significant adverse effects in normal tissues surrounding the tumor.

To our knowledge, the efficacy and bioavailability of allicin in a NB tumor mouse model has not been studied before. Allicin, if taken orally, is hydrolyzed in the acidic environment in the stomach and in addition it reacts with GSH in cells and tissues, further reducing the amount of allicin that might reach tumors to *S*-thioallylate cysteine residues there. In regard of these factors reducing the bioavailability of allicin taken orally, we reasoned that a more effective means of administration for allicin would be direct intra-tumoral injection. Our results confirmed that allicin administered in this way is quite potent and indeed significantly reduced NB PDX tumor growth *in vivo* (Figure 3).

Conclusion

There is increasing interest in, and new technologies available for, the treatment of cancer patients *via* intra-tumoral injections, an administration strategy that has been successful in the treatment of *in vivo* cancer models (47). Moreover, this tumor-tissue targeted treatment method also significantly reduces the occurrence of side effects commonly associated with systemic chemotherapy, because allicin is liable to be largely titrated out within the tumor itself by reacting with its substrates *in situ*. Thus, for these reasons, allicin, or perhaps allyl polysufanes that are able to carry out the thioallylation of thiols, might be premier candidates for intra-tumoral treatments of tumors including neuroblastoma, glioblastoma, and medulloblastoma.

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Authors' Contributions

ASB designed the study and developed the technical protocols. MCHG synthesized allicin. CRS performed all biological experiments including PDX *in vivo* studies, evaluated raw data, and prepared final figures. ASB, CRS, and AJS interpreted data and wrote the manuscript. All Authors reviewed the final draft.

Conflicts of Interest

The Authors report no conflicts of interest in relation to this study.

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