Evaluation of the STAT3 inhibitor GLG-302 for the prevention of estrogen receptor-positive and -negative mammary cancers

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Abstract. Signal transducer and activator of transcription 3 (STAT3) plays a key role in the transformation of normal cells to cancerous cells. Although inhibitors of STAT3 have been shown to suppress the growth of multiple cancer types *in vitro* and *in vivo,* such agents are of particular interest for the prevention of breast cancer, which affects over 200,000 women and claims more than 40,000 lives in the United States each year. In the present study, we employed the MMTV/Neu transgenic mouse model, which develops estrogen receptor (ER)-negative, Neu-overexpressing tumors, and the Sprague-Dawley (SD) rat model, which develops ER-positive tumors upon exposure to the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA), to test the efficacy of the STAT3 inhibitor GLG-302 in the prevention of mammary cancer. Orally administered GLG-302 and its trizma salt derivative reduced mammary cancer incidence, multiplicity, and tumor weights in female MMTV/Neu mice, and GLG-302 reduced tumor multiplicity and weights in female DMBA-treated rats. Consistent with the mechanism of action of STAT3 inhibitors, the reductions in mammary tumors were correlated with decreases in STAT3 phosphorylation and cell proliferation. These data suggest that GLG-302 is a novel agent with potential for prevention of mammary cancer and support the further development of STAT3 inhibitors for this cause.

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Abbreviations: DCIS, ductal carcinoma *in situ*; DMBA, 7,12-dimethylbenz[a]anthracene; EGFR, epidermal growth factor receptor; ER, estrogen receptor; HER2/Neu, human epidermal growth factor receptor 2; IL6-R, interleukin 6 receptor; JAK, Janus kinase; PDGFR, platelet-derived growth factor receptor; PIAS, protein inhibitor of activated STAT; PR, progesterone receptor; RXR, retinoid X receptor; SD, Sprague-Dawley; SERM, selective estrogen receptor modulator; SH2, src-homology 2; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; VEGFR, vascular endothelial growth factor receptor

Key words: STAT-3, GLG-302, mammary cancer, prevention

Introduction

Cancer chemoprevention involves the use of natural, synthetic, or biological agents to suppress or delay the initial phases of carcinogenesis or the progression of premalignant cells to invasive disease. These agents are administered to individuals without overt disease who harbor pre-cancerous lesions or who are genetically predisposed to developing cancer. Because chemopreventive drugs are administered to generally healthy individuals over a long period of time, they must exhibit little-to-no toxicity. This requirement has been a longstanding barrier to moving chemopreventive agents to the clinic; and, as a result, the FDA has only approved a very small number of agents for cancer risk reduction across all subtypes of cancer (1). For breast cancer prevention, the selective estrogen receptor modulators (SERMs) tamoxifen and raloxifene have been approved for use in women at high risk for the disease, and in women with ductal carcinoma *in situ* (DCIS) who have undergone breast surgery and radiation. However, despite their proven efficacy, these drugs have failed to gain acceptance from patients and health care providers due to their potential to cause hot flashes, induce thromboembolic events, and increase the risk of uterine cancer (2,3). Thus, there is a critical need for the identification and development of new chemopreventive agents for breast cancer.

The molecular targets for cancer chemoprevention include factors involved in DNA damage/repair, inflammation, cellular metabolism, apoptosis, angiogenesis, and signal transduction. One such factor is signal transducer and activator of transcription 3 (STAT3). STAT3 is one of the seven members of a family of transcription factors that regulates cell proliferation, differentiation, apoptosis, and the immune response. Upon ligand binding, cytokine and growth factor receptors such as the IL6 receptor (IL6-R), epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), and platelet-derived growth factor receptor (PDGFR) dimerize, resulting in the recruitment and subsequent activation of Janus kinases (JAKs). Activated JAKs in turn phosphorylate tyrosine residues on the cytoplasmic domain of the receptor,

creating a docking site for the src-homology 2 (SH2) domain of STAT3 and enabling the phosphorylation and activation of the STAT3 protein (4). Upon activation, STAT3 dimerizes via its SH2 domain and translocates to the nucleus where it promotes the expression of numerous target genes involved in cell proliferation and survival [cyclin D1 (5), c-myc (6), Bcl-X_L (7), survivin (8)], migration and invasion [MMPs (9)], angiogenesis [VEGF (10), HIF-1 (11)], and immune suppression [TGFβ, IL-10 (12)]. STAT3 can also be activated in a receptor-independent manner by the Src and Abl kinases (4).

In normal cells, the activation of STAT3 is transient and is highly regulated by phosphatases, ubiquitinases, and the suppressor of cytokine signaling (SOCS) and protein inhibitor of activated STAT (PIAS) proteins (4). However, in many types of cancer, including breast (13), ovarian (14), prostate (15), colon (16), renal (17), brain (18), and pancreatic cancer (19), STAT3 is constitutively active. This correlation, combined with the findings that transgenic mice expressing constitutively active STAT3 exhibit an increased rate of tumor formation and a greater tumor burden than their wild-type counterparts (20,21), and that the reduction or inactivation of the STAT3 protein prevents transformation and promotes apoptosis in animal models of cancer (22,23), supports a role for STAT3 in carcinogenesis and suggests that STAT3 could serve as a target for preventive intervention.

Targeting STAT3 is especially appealing for the prevention of breast cancer. STAT3 is constitutively active in over 40% of all breast cancers, particularly in triple-negative breast cancers which lack the expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/Neu) (24). Activated STAT3 has also been shown to induce estrogen biosynthesis and the subsequent proliferation of ER-positive breast epithelial cells (25), and is thought to play a role in the maintenance of tumor recurrence-promoting stem cell-like breast cancer cells and in the conversion of a non-cancer stem cell population to breast cancer stem cell-like cells (26). Thus, STAT3 inhibitors offer a unique advantage over the FDA-approved breast cancer preventive agents tamoxifen and raloxifene in that they could potentially prevent multiple breast cancer subtypes. In addition, because STAT3 inhibitors have a distinct mechanism of action from the SERMs tamoxifen and raloxifene, such inhibitors may also be particularly useful against ER-positive breast cancers that have developed resistance to these drugs.

GLG-302 (S3I-201, NSC 74859) is a STAT3 inhibitor that was identified through docking simulations that relied on the X-ray crystal structure of the STAT3β homodimer bound to DNA to screen the National Cancer Institute's chemical libraries (27). GLG-302 is an inhibitor of STAT3 DNA-binding activity *in vitro* with an IC_{50} of 86±33 μM (although it also shows low activity toward STAT1 and STAT5), and it suppresses the growth of cells containing constitutively active STAT3 (27-29). Previous studies have shown that treatment with GLG-302 induces apoptosis in breast cancer cell lines through the repression of STAT3-mediated cyclin D1, Bcl-xL, and survivin expression, and that it can inhibit the growth of pre-established breast cancer tumors in xenograft mouse models (27).

In the present study, we investigated the ability of orally-administered GLG-302 and its trizma salt derivative to prevent the development of mammary cancers in female MMTV/Neu mice and 7,12-dimethylbenz[a]anthracene (DMBA)-exposed Sprague-Dawley (SD) rats. The MMTV/Neu ($ErbB2^{+/-}$) model of breast cancer was initially developed by Muller and colleagues (30-32). It employs the overexpression of wild-type Neu and develops ER-negative mammary carcinomas that overexpress Neu. The absence of the ER and the overexpression of wild-type Neu are characteristics of approximately 15% of all human breast cancers. There is evidence that the expression of STAT3 is modulated by EGFR family members including Neu (EGFR2) (24), and preliminary studies in our laboratory demonstrated that activated STAT3 was present in the normal mammary tissue of female MMTV/Neu mice (unpublished data). The DMBA-induced rat model of breast cancer was first described by Huggins *et al* in 1961 (33). Similar to approximately 70% of all human breast cancers, rat mammary tumors that arise following a single dose of the carcinogen DMBA are ER-positive and PR-positive, and are thus strongly hormone dependent. Furthermore, our laboratory has shown that activated STAT3 is highly expressed in the mammary tissue of these female animals. Taken together, these data indicate that Neu-overexpressing and DMBA-induced tumors are good candidates for testing the efficacy of a STAT3 inhibitor, and support the selection of the MMTV/Neu and DMBA-treated SD models to evaluate the chemopreventive activity of GLG-302.

Materials and methods

Female MMTV/Neu ($ErbB2^{+/-}$) mice were generated in the Chemoprevention Center at the University of Alabama at Birmingham by crossing $ErbB2^{+/+}$ mice with female FVB mice. Mice were genotyped by tail clips prior to being placed on test. Female Sprague-Dawley rats were obtained from Envigo (Madison, WI, USA) at 28 days of age. Animals received Teklad (4% fat) diet purchased from Envigo. All mice were housed (5/cage) in a room artificially lighted 12 h/day and maintained at 23±2˚C. Access to food and water was *ad libitum*. All mice with large tumors (as defined by IACUC guidelines) were sacrificed. GLG-302 was provided by GLG Pharma (Jupiter, FL, USA). The high doses of GLG-302 used resulted in a fairly dense mixture that created difficulties during the gavage process. In order to improve drug handling and delivery and to increase the bioavailability and efficacy of the compound, GLG-302 was reformulated so that it could be dissolved in an aqueous medium. Several derivatives of GLG-302 were synthesized, and a trizma salt form of the compound (GLG-302/trizma salt) was ultimately selected because it was water soluble at concentrations as high as 50 mg/ml and it displayed acceptable stability when frozen until administered to the animals. GLG-302/trizma salt was provided by the National Cancer Institute/Division of Cancer Prevention Chemical Repository. For the mouse studies, the vehicle for GLG-302 was 0.5% carboxymethylcellulose (pH 6.5), and the vehicle for GLG-302/trizma salt was water. For both agents, the volume was 0.2 ml/mouse, and the agents were given daily, 5x/week. For administration of GLG-302 to the rats, the agent was incorporated with the diet using a Patterson-Kelly blender with intensifier bar. Fresh diet was provided to the rats 3x/week. 7,12-Dimethylbenz[a]

anthracene (DMBA) was purchased from Sigma-Aldrich Corporation/Merck KGaA. For the mice, DMBA was dissolved in corn oil and administered by gavage (0.2 ml). For the rats, 1.0 ml of the DMBA solution was given. All animal experiments were conducted in AAALAC-approved facilities following procedures approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham (project number IACUC-20269). All animals were weighed 1x/week and palpated for mammary tumors 2x/week. At the end of the study, animals were sacrificed using CO_2 asphyxiation followed by a double pneumothorax. All mammary tumors were evaluated by a board-certified pathologist (MMJ) and weighed.

Mouse chemoprevention studies. In the first study, female mice (15/group) were randomized into the following groups: GLG-302 [500 mg/kg body weight (BW)/day], GLG-302 (250 mg/kg BW/day), GLG-302 (125 mg/kg BW/day) and no treatment. The doses were selected based on a 3-month maximun tolerated dose (MTD) study (Table SI), which revealed no signs of toxicity or changes in animal body weight. (Table SII). For the chemoprevention studies, the mice received the agents beginning at 65 days of age and continuing for the duration of the study (10 months of treatment).

In the second study, female mice (25/group) were randomized as follows: GLG-302/trizma salt (500 mg/kg BW/day), GLG-302/trizma salt (250 mg/kg BW/day), GLG-302/trizma salt (125 mg/kg BW/day), and no treatment. These doses were also selected based on a previous six-week study in our laboratory. The mice received the agents beginning at 50 days of age and continuing for the duration of the study. DMBA was initially given at 57 days of age (1x/week for 4 weeks) to accelerate tumor development. The study was terminated 19 weeks after the initial DMBA treatment. All deaths in both studies were due to gavage errors.

Rat chemoprevention study. Female rats (15/group) were randomized into the following groups: GLG-302 (8 g/kg diet) and no treatment. The rats received the agents beginning at 43 days of age and continuing for the duration of the study. DMBA was administered at 50 days of age (50 mg/kg BW by gavage). The study was terminated 126 days after DMBA treatment.

Proliferation and p-STAT3 measurements. In separate studies, the effects of GLG-302 and GLG-302/trizma salt on normal mammary epithelial cell proliferation and p-STAT3 levels were evaluated. Beginning at 7-8 weeks of age, MMTV/Neu mice or SD rats (5/group) received the STAT3 inhibitors for 2 weeks by gavage (5x/week). For GLG-302, dose levels of 500, 200 and 100 mg/kg BW/day were administered to the mice, while for GLG-302/trizma salt, dose levels of 500, 250, and 125 mg/kg BW/day were given. The rats received 500 mg/kg BW/day GLG-302. All animals were sacrificed one day after the last treatment with the agents. Mammary tissue was excised from an area in the abdominal/inguinal glands (adjacent to the linea alba) that contains a high concentration of epithelial cells. Mammary cancers from mice were excised at the end of the study that used GLG-302/trizma salt. The mammary tissues were fixed in 10% formalin for 24 h at room temperature and were then transferred to 70% ethanol until histologically processed.

After embedding in paraffin blocks, sections (4-μm thick) were placed on positive microscope slides. The tissues were de-paraffinized with xylene and placed in ethanol. Antigen retrieval employed boiling in sodium citrate (pH 6.0) for 20 min. Slides were then covered with peroxidase block for 3 h and washed with Tris buffer. The tissues were incubated with primary antibody p-STAT3 (cat no. 9145S; Cell Signaling Technology, Danvers, MA, USA) or Ki-67 (cat. no. AB1667; Abcam, Cambridge, MA, USA) for 1 h at room temperature. The dilution factor for p-STAT antibody was 1:200, while that for Ki-67 was 1:100. Processing and staining of tissue were performed according to the manufacturer's procedures (DAKO Envision + Kits; Agilent Technologies, Inc.). Tissues were then washed and dehydrated in ethanol and xylene. The images were captured and counted using the Aperio Scan Scope imaging system (Aperio Imaging, Visa, CA, USA). For counting the cells, each area containing mammary ductal epithelial cells was randomly analyzed (stained cells + total cells counted) by a program within ScanScope. A total of 1,000-1,500 cells were usually counted. This varied depending on the degree of proliferation resulting from treatment of the animal with the agent.

Statistical analysis. Final mammary tumor incidence was compared using Chi-square or Fisher's exact tests. Mammary cancer latency was analyzed with a Kaplan-Meier estimate and compared with a log-rank test. Tumor multiplicity was compared using a Cochran-Armitage trend test for mouse studies and Poisson regression for the rat study. Tumor weights were analyzed via Chi-square for mouse studies and Mann-Whitney U test for the rat study. Proliferation indices were analyzed using one-way ANOVA. Due to the longitudinal nature of the studies, experiments were not duplicated. Data are presented as mean ± standard error. All statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). $P<0.05$ was assigned as indicative of a statistically significant difference.

Results

Effect of GLG-302 on spontaneous mammary cancers in female MMTV/Neu mice. GLG-302 was evaluated at various doses for efficacy in the prevention of spontaneous mammary cancers occurring in female MMTV/Neu mice. There were no gross signs of toxicity, and the body weights of the mice were not significantly altered during the study. Because of the viscosity of the GLG-302 mixture (particularly at the higher doses), several deaths occurred in the various groups due to gavage errors (i.e., not related to drug toxicity). GLG-302 at dose levels of 500 and 250 mg/kg BW/day significantly decreased mammary cancer multiplicity by 55 and 46%, respectively (Table IA and Fig. 1A). The highest dose also significantly reduced the weight of the mammary cancers (77%) (Table IA). The effects of short-term (2-week) treatment with various doses of GLG-302 on the rate of proliferation of normal mammary epithelial cells is shown in Fig. 2A. Only the highest dose (500 mg/kg BW/day) was significantly effective

Table I. Effect of GLG-302 (STAT3 antagonist) on mammary tumor incidence, multiplicity, and weight in female MMTV/Neu mice and DMBA-treated SD rats.

A, MMTV/Neu mice

GLG-302 (mg/kg BW/day)	Incidence (%)	Multiplicity (tumors/mouse)	Tumor weight (g)
500	60 (25%↓)	1.00 (55%↓)[a]	0.40 (77%↓)[a]
250	64 (20%↓)	1.18 (46%↓)[a]	1.26 (28%↓)
125	85 (6%↑)	1.69 (23%↓)	1.50 (14%↓)
0	80	2.20	1.75

The number in parenthesis is the percent increase (↑) or decrease (↓) from the control (0 mg/kg BW/day) group (n=10-15/group). [a]P<0.05 compared to the control group.

B, DMBA-treated SD rats

GLG-302 (g/kg diet)	Incidence (%)	Multiplicity (tumors/rat)	Tumor weight (g)
8	47 (11%↓)	0.87 (52%↓)[b]	0.14 (87%↓)[b]
0	53	1.80	1.10

The number in parenthesis is the percent decrease (↓) from the control (0 g/kg diet) group (n=15 rats/group). [b]P≤0.1 compared to the control group. DMBA, 7,12-dimethylbenz[a]anthracene.

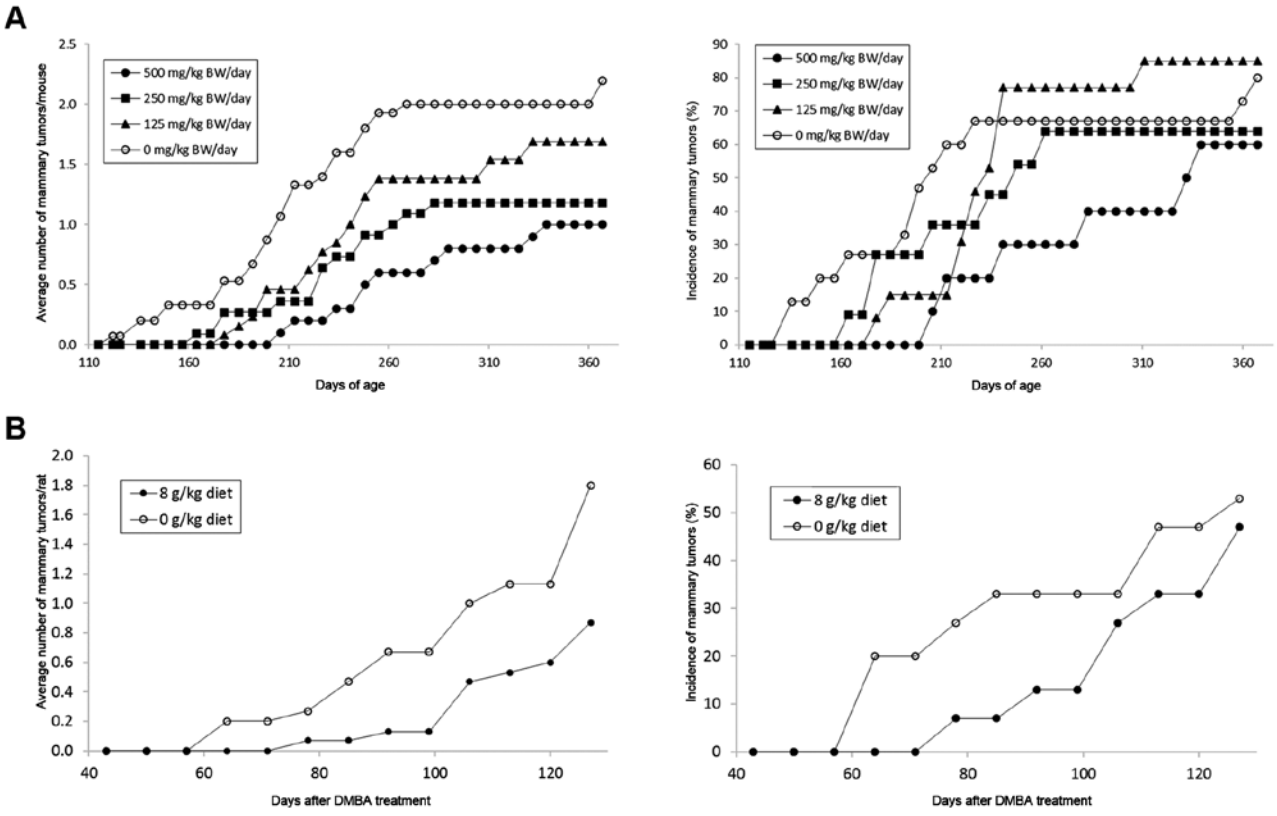

Figure 1. Effect of GLG-302 on the appearance of spontaneous mammary cancers in female MMTV/Neu mice and DMBA-treated rats. (A) When compared to the control group, none of the treatment groups in the MMTV/Neu mouse study (n=10-15/group) showed a significant difference in tumor latency. (B) For female Sprague-Dawley rats (n=15/group), there were no significant differences in tumor latency. The statistical analyses of the tumor multiplicity and incidence at the end of the studies are documented in Tables IA and B. DMBA, 7,12-dimethylbenz[a]anthracene; GLG-302, STAT3 inhibitor.

in reducing the proliferation index. The effect of the highest dose of GLG-302 on normal epithelial cell proliferation was correlated with the significant effect of this dose in the prevention of mammary cancers.

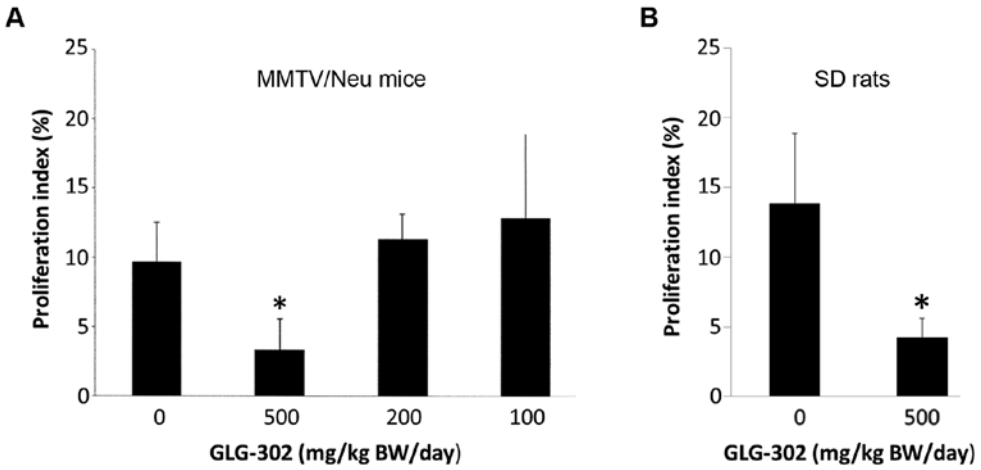

Figure 2. Effect of GLG-302 on the rate of proliferation of epithelial cells in the normal mammary glands of female MMTV/Neu mice and Sprague-Dawley rats. (A) Seven to eight-week old MMTV/Neu mice or (B) Sprague-Dawley rats were treated with different doses of GLG-302 by gavage. The error bars represent SEM. *P<0.05, a statistically significant difference from the control group. GLG-302, STAT3 inhibitor.

Effect of GLG-302 on carcinogen-induced mammary cancers in female Sprague-Dawley rats. A single dose of GLG-302 was also evaluated for efficacy in the prevention of spontaneous mammary cancers occurring in female DMBA-treated rats. GLG-302 was administered in the diet for 4 months, beginning one week prior to carcinogen treatment at 50 days of age. Rats fed 8 g GLG-302/kg diet displayed no gross signs of toxicity and maintained body weights comparable to vehicle-treated rats throughout the study period (data not shown). Although statistical significance levels were lower than our usual standard (P<0.05) due to the unexpectedly low yields of tumors in control animals and consequent effects on standard deviations and small absolute differences, treatment with GLG-302 resulted in a 52% decrease in tumor multiplicity (P=0.1) and an 87% decrease in tumor weight (P=0.07) (Table IB and Fig. 1B). In agreement with this data, the short-term (2-week) administration of 500 mg/kg BW/day GLG-302 by gavage reduced the rate of proliferation of normal mammary epithelial cells by 80% (Fig. 2B).

Effect of GLG-302/trizma salt on mammary cancers in female MMTV/Neu mice. The examination of the pharmacokinetic properties of GLG-302 indicated that the absorption of the compound was sub-optimal. We therefore developed a trizma base formulation (referred to as GLG-302/trizma salt) and evaluated its chemopreventive activity in MMTV/Neu mice. Because the previous study had resulted in a relatively long tumor latency period in untreated mice (a 50% incidence of palpable lesions was not obtained until 206 days of age in the untreated control group), the mice in this study received DMBA beginning at 57 days of age to reduce the length of the study from approximately 10 months to 4 months. As in the previous mouse study, the mice received the STAT3 inhibitor by gavage, but beginning one week prior to the carcinogen. As with GLG-302, treatment with the trizma salt derivative resulted in a dose-dependent decrease in the multiplicity of mammary cancers; significant decreases of 72 and 41% were observed at the 500 and 250 mg/kg BW/day dose levels, respectively (Table II and Fig. 3). The two highest doses also decreased the weight of the mammary cancers by approximately 70-80% (Table II), and the 500 mg/kg BW/day dose significantly increased tumor latency (P=0.012) and reduced tumor incidence by 53% (Table II and Fig. 3). The GLG-302/trizma salt did not alter body weights or induce gross toxicity during the study (data not shown).

The epithelial cells of the normal mammary glands of female MMTV/Neu mice showed decreased proliferation after two weeks of treatment with GLG-302/trizma salt as determined by Ki-67 staining (Fig. 4A). In contrast to what was observed with GLG-302 (Fig. 2), the two highest doses of GLG-302/trizma salt both resulted in over 80% reductions in the proliferation index (Fig. 4A), suggesting that the new formulation may be more potent than its parent compound. The normal mammary epithelial cells also showed dose-dependent decreases in phosphorylated STAT3 (p-STAT3) levels after two weeks of treatment with GLG-302/trizma salt (Fig. 4B), suggesting that the chemopreventive effects of GLG-302/trizma salt were associated with STAT3 inhibition and a subsequent decrease in cell proliferation. To confirm this, we measured Ki-67 and pSTAT3 in the tumors harvested from the mice upon completion of the study. In agreement with the lower tumor weights of the GLG-302-treated animals and the staining results in the normal mammary glands, Ki-67 (Fig. 4C) and p-STAT3 (Fig. 4D) were significantly reduced in the cancers of the mice that had received 500 mg/kg BW/day GLG-302/trizma salt for 4 months.

Discussion

In the present study, we provide support for the use of STAT3 inhibitors as breast cancer chemopreventive agents by demonstrating that GLG-302 has an effect on cancer formation in the ER-negative, Neu-overexpressing MMTV/Neu mouse and DMBA-induced ER-positive rat models of breast cancer by preventing STAT3 activation and subsequently reducing cell proliferation. The doses of GLG-302 used in this study are those that can be achieved in the human. For example, using a standard conversion factor that converts mg/kg in mouse to mg/kg in humans, one multiplies the dose of 500 mg/kg

Table II. Effect of GLG-302/trizma salt on mammary tumor incidence, multiplicity and weight in female DMBA-treated MMTV-Neu mice.

GLG-302/trizma salt (mg/kg BW/day)	Incidence (%)	Multiplicity (tumors/mouse)	Tumor weight (g)
500	33 (53%↓)[a]	0.61 (72%↓)[a]	0.24 (81%↓)[a]
250	59 (16%↓)	1.27 (41%↓)[a]	0.37 (71%↓)[a]
125	63 (10%↓)	2.11 (3%↓)	1.03 (20%↓)
0	70	2.17	1.28

The number in parenthesis is the percent decrease (↓) from the control (0 mg/kg BW/day) group (n=18-23 mice/group). [a]P<0.05 compared to the control group. BW, body weight; DMBA, 7,12-dimethylbenz[a]anthracene; GLG-302, STAT3 inhibitor.

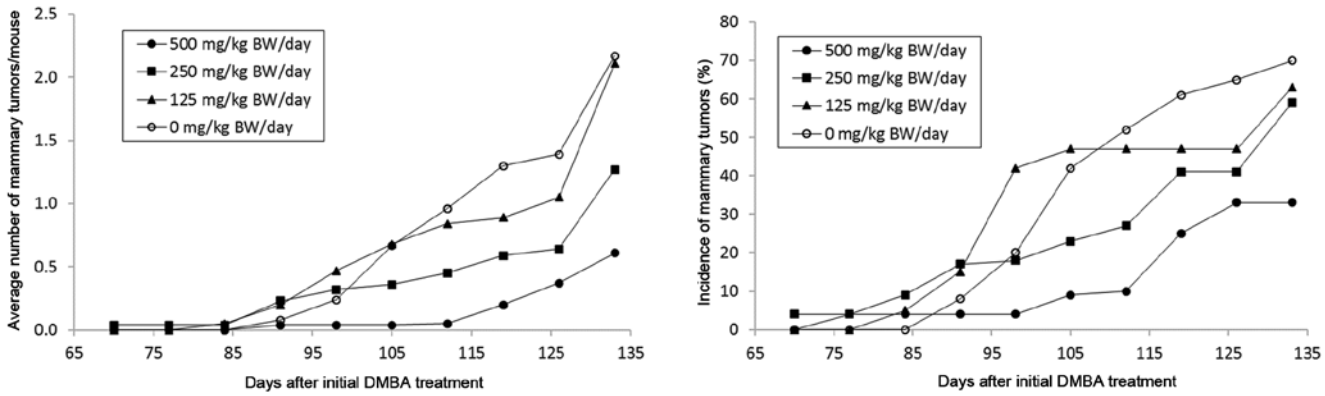

Figure 3. Effect of GLG-302/trizma salt on the appearance of DMBAinduced mammary cancers in female MMTV/Neu mice. When compared to the control group, only the highest (500 mg/kg BW/day) treatment group showed a significant difference in tumor latency (P=0.012). The statistical analyses of tumor multiplicity and incidence at the end of the study are described in Table II. n=18-23/group. DMBA, 7,12-dimethylbenz[a]anthracene; GLG-302, STAT3 inhibitor.

BW/day in the mouse by 0.08 to obtain a dose of 40 mg/kg BW for a human. Thus, this dose can have clinical significance.

Although distinct from triple-negative and *BRCA1* mutant mammary cancers, the MMTV/Neu mouse is clearly a relevant model for Neu-overexpressing breast cancer in humans. Neu-overexpressing tumors account for approximately 15% of total human breast tumors, and although they are most commonly ER-negative, they also represent a significant percentage of ER-positive (Luminal B) tumors that are highly proliferative, have low ER levels, and fail to respond to hormonal agents such as SERMs and aromatase inhibitors (34-36). Furthermore, Neu-overexpressing tumors comprise a high percentage of pre-invasive DCIS lesions, which are potential targets for prevention studies (37,38). The results achieved here with GLG-302 and GLG-302/trizma salt in this model are profound and reduce mammary cancer multiplicity to approximately the same extent as EGFR inhibitors and retinoid X receptor (RXR) agonists, which have heretofore been the most effective agents in this model (39,40).

In terms of histology, the carcinogen-treated rat model is most relevant to hormone receptor-positive breast cancers, which make up 70% of all cases of the disease; approximately 76% of the mammary cancers in the DMBA-treated rat model are of the Luminal A subtype, whereas 24% have been characterized as Luminal B (41). Since ER and PR expression is found in the majority of DCIS lesions (42), the carcinogen-induced rat has become one of the most commonly used animal models for breast cancer chemoprevention studies. Although the preventive efficacy of GLG-302/trizma salt could not be determined due to the failure of DMBA to sufficiently induce tumors in the control animals (data not shown), GLG-302 showed a distinct trend toward reducing tumor multiplicity and weight in this model, despite its poor bioavailability.

Over the years, many other STAT3 inhibitors in addition to GLG-302 have been identified and developed. These include compounds that directly prevent STAT3 phosphorylation, dimerization, translocation, or DNA binding by targeting the SH2, N-terminal, or DNA binding domains of the protein, and compounds that indirectly interfere with STAT3 activity by blocking its upstream regulators (43,44). Although potentially useful in a chemotherapeutic setting where compound toxicity is generally tolerated as a trade-off for increased patient survival, most of these STAT3 inhibitors are not suitable for the prevention of cancer in healthy individuals, as they produce numerous side effects including fatigue, nausea, diarrhea, anemia, and infection. It remains to be determined if the employment of GLG-302 as a clinical chemopreventive agent will be hampered by the same side effects that have restricted the use of many other STAT3 inhibitors. However, our preclinical studies suggest that this will not be the case, since as much as 500 mg GLG-302/kg BW/day did not significantly affect the body weights of the animals or induce other signs of toxicity during this study. Similarly, GLG-302 was well-tolerated in rats and dogs in pilot safety studies conducted by GLG Pharma.

The design of and testing of new GLG-302 analogs is currently underway to improve agent efficacy. Several of these

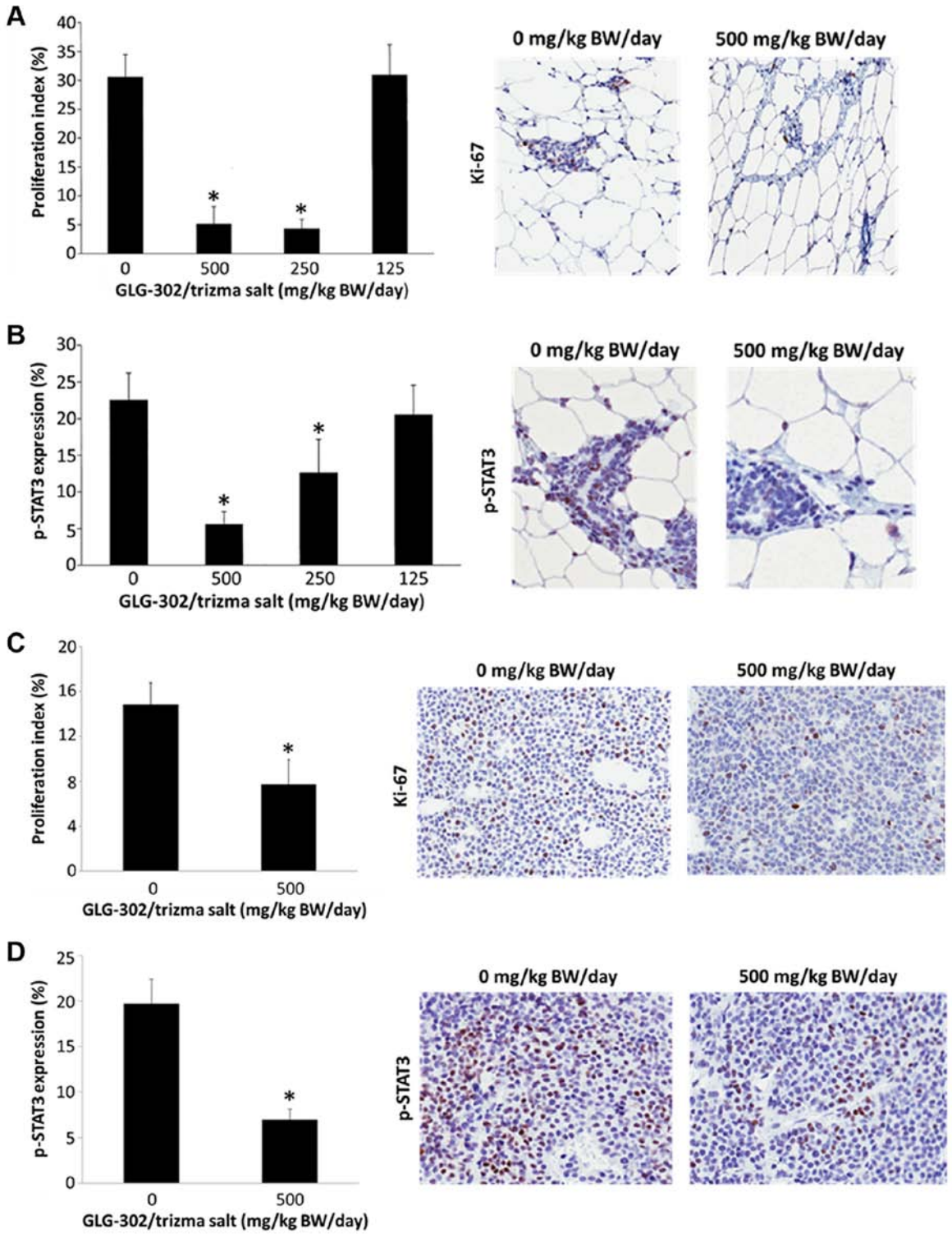

Figure 4. Effect of GLG-302/trizma salt on the rate of proliferation and p-STAT3 expression in the normal epithelial cells of female MMTV/Neu mice and in mammary cancers excised from female MMTV/Neu mice. (A and B) Seven- to eight-week old mice (n=3-4/group) were treated with different doses of GLG-302 by gavage. Mammary tissue was analyzed for (A) Ki-67 and (B) p-STAT3 expression. (C and D) Tumors (n=5/group) excised from the mice in the 500 mg/kg BW/day treatment group in Fig. 3 were analyzed for (C) Ki-67 and (D) p-STAT3 expression. In all panels, error bars represent SEM. *P<0.05, a statistically significant difference from the control group. Representative images used for the quantification are shown to the right of each graph (magnification, x20). GLG-302, STAT3 inhibitor; p-STAT3, phosphorylated signal transducer and activator of transcription 3.

derivatives, including S3I-201.1066, BP-1-102, S3I-1757, and SH5-07, have improved potencies compared to GLG-302 and have shown activity *in vitro* and in xenograft models of breast cancer (45-48). However, further studies are needed to determine if these small molecules, similar to their parent compound GLG-302, could also be used as chemopreventive agents.

It is also feasible that GLG-302 or its derivatives could be used in combination with other agents that act synergistically or additively with the STAT3 inhibitor to further suppress

mammary tumor development. For example, GLG-302 has been shown to act synergistically with metformin to decrease cell growth and induce apoptosis in triple-negative breast cancer cell lines (49). Although some success has been achieved in the area of combinatorial chemoprevention (50), toxic interactions between the agents are a serious concern. A safer and more effective alternative may be to use GLG-302 in combination with a cancer vaccine to directly inhibit tumor development while simultaneously altering the immunologic environment in favor of immunoprevention.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Author's contributions

RHS and CJG designed the experiments. CJG, MMJ, FLM and JTF performed the experiments and/or analyzed the data. RHS, CJG and JTF wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All animal experiments were conducted in AAALAC-approved facilities following procedures approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham (project number IACUC-20269).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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