Video Article

Synthesis and Characterization of an Aspirin-fumarate Prodrug that Inhibits NFκB Activity and Breast Cancer Stem Cells

Irida Kastrati[1], Loruhama Delgado-Rivera[2], Gergana Georgieva[3], Gregory R. J. Thatcher[2], Jonna Frasor[1]

[1]Physiology and Biophysics, College of Medicine, University of Illinois at Chicago

[2]Medicinal Chemistry and Pharmacognosy, College of Medicine, University of Illinois at Chicago

[3]College of Pharmacy, University of Illinois at Chicago

Correspondence to: Irida Kastrati at ikastr2@uic.edu

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Abstract

Inflammation is a cancer hallmark that underlies cancer incidence and promotion, and eventually progression to metastasis. Therefore, adding an anti-inflammatory drug to standard cancer regiments may improve patient outcome. One such drug, aspirin (acetylsalicylic acid, ASA), has been explored for cancer chemoprevention and anti-tumor activity. Besides inhibiting the cyclooxygenase 2-prostaglandin axis, ASA's anti-cancer activities have also been attributed to nuclear factor κB (NFκB) inhibition. Because prolonged ASA use may cause gastrointestinal toxicity, a prodrug strategy has been implemented successfully. In this prodrug design the carboxylic acid of ASA is masked and additional pharmacophores are incorporated.

This protocol describes how we synthesized an aspirin-fumarate prodrug, GTCpFE, and characterized its inhibition of the NFκB pathway in breast cancer cells and attenuation of the cancer stem-like properties, an important NFκB-dependent phenotype. GTCpFE effectively inhibits the NFκB pathway in breast cancer cell lines whereas ASA lacks any inhibitory activity, indicating that adding fumarate to ASA structure significantly contributes to its activity. In addition, GTCpFE shows significant anti-cancer stem cell activity by blocking mammosphere formation and attenuating the cancer stem cell associated $CD44^{+}CD24^{-}$ immunophenotype. These results establish a viable strategy to develop improved anti-inflammatory drugs for chemoprevention and cancer therapy.

Video Link

The video component of this article can be found at https://www.jove.com/video/54798/

Introduction

Inflammation is a hallmark that underlies multiple aspects of tumorigenesis, such as incidence and promotion, and eventually progression to metastasis[1]. In breast cancer, this is further supported by epidemiological observations showing that regular use of the classical non-steroidal anti-inflammatory drug aspirin (acetyl salicylic acid, ASA) is associated with a reduction in both breast cancer incidence, and risk of metastasis and recurrence[2,3]. ASA acts primarily by inhibiting cyclooxygenase-2 activity, which is often upregulated in breast cancer[4,5]. However, the anti-cancer effects of ASA may also be mediated by suppressing aberrant nuclear factor κB (NFκB) signaling[6-8]. This is important because a deregulated NFκB pathway promotes tumor cell survival, proliferation, migration, invasion, angiogenesis, and resistance to therapy[9-11]. NFκB pathway activation is also critical for mounting an immune response. Therefore, for anti-cancer therapy where prolonged NFκB inhibition is required, one must consider the detrimental side effects involving long-lasting immune suppression. Hence, ASA may serve as a good starting point for therapeutic optimization.

One limitation for ASA application in cancer therapy is the elevated doses required for cyclooxygenase 2 and NFκB inhibition, which are associated with gastrointestinal toxicity, such as ulcers and stomach bleeding[12,13]. However, converting ASA into as ester prodrug, may reduce ASA's gastrointestinal toxicity. To further enhance potency and/or add functionality, additional structural elements or ancillary pharmacophores may also be incorporated into ester prodrug design. One such pharmacophore added to enhance ASA potency against the NFκB pathway is fumarate, which we have previously shown to be important for NFκB pathway inhibition[14,15].

We synthesized an aspirin-fumarate prodrug[15], GTCpFE, and hypothesized that such hybrid molecule would be safe yet potent against the NFκB pathway. We tested its anti-NFκB activity in breast cancer cells and its ability to block breast cancer stem cells (CSCs)[15], which rely on NFκB signaling for survival and growth[16-21]. We find that the potency of GTCpFE against the NFκB pathway is significantly improved over ASA[15]. In addition, GTCpFE blocks mammosphere formation and attenuates the CSC surface marker $CD44^{+}CD24^{-}$ immunophenotype, indicating that GTCpFE is capable of eradicating CSCs[15]. These results establish the aspirin-fumarate prodrug as an effective anti-inflammatory agent that can also target breast CSCs. In terms of breast cancer therapy, GTCpFE may have the potential to treat aggressive and deadly disease.

Protocol

1. Synthesis of Aspirin-fumarate Prodrug GTCpFE

1. Using a plastic plunger syringe, measure 0.81 ml (20 mmol) of methanol and mix it in water (10 ml) in a round bottom flask. Cool the resulting mixture to 0 °C by placing the flask in an ice-water bath. Add 4-hydroxybenzyl alcohol (2.48 mg, 20 mmol) and stir the reaction mixture until the solution is clear.
2. Prepare a solution of *O*-acetylsalicyloyl chloride (3.77 mg, 19 mmol) in anhydrous toluene (10 ml) by weighing the desired amount of *O*-acetylsalicyloyl chloride and dissolving it in the solvent in a separate flask. Using a plastic plunger syringe, add this solution to the mixture prepared in step 1.1, and leave the reaction stirring at 0 °C.
3. Monitor the reaction using thin layer chromatography (TLC). The TLC plates should have silica as a stationary phase.
    1. Prepare a mobile phase composed of 20:80 ethyl acetate (AcOEt)/hexane. In a TLC chamber (or small container) add 5 ml of mobile phase to cover the bottom of the chamber.
    NOTE: The amount of mobile phase depends on the chamber selected. Always add enough to cover the bottom, but once the TLC is placed inside, the solvent line should not surpass the height where the compounds are spotted.
    2. Take a sample of the reaction with a syringe (0.2 ml), place it in a small vial, and dilute it with ethyl acetate (2-3 drops). With a TLC spotter, carefully take a sample of the diluted reaction mixture and spot it in the TLC.
    NOTE: The spots should be 1-2 mm and this should be done at the lower 1/4 inch of the TLC plate.
    3. On the same TLC, place a spot of a solution of O-acetylsalicyloyl chloride (0.2 mg in 0.5 ml of ethyl acetate) as a comparison. Once the spots are dry, place it in the chamber and let it run until the solvent front has almost reached the top of the TLC.
    4. Take the TLC out, let it dry and visualize it under a UV lamp. The reaction is complete when the *O*-acetylsalicyloyl chloride spot disappears from the reaction mixture.
    5. When the reaction is completed, remove the ice-water bath, and allow the reaction to stir at room temperature for 20 hr.
4. Filter the precipitate using a Buchner funnel with a fritted disk of medium porosity. Place the solid in a scintillation vial, and leave the compound *in vacuo* overnight in a desiccator at room temperature. Use phosphorus pentoxide (P_2O_5) as the desiccant.
5. Dry a round bottom flask by placing it in an oven at 80 °C for at least two days before setting up this reaction. Alternatively, seal the flask with a septum and pierce it with a needle that is connected to a vacuum pump system. Turn the vacuum pump on and dry the flask using a heat gun. Allow to cool, and repeat this step 2 more times.
6. Inflate a balloon using argon gas, and connect it to a plastic syringe (whose plunger has been removed) with a needle. Turn off the vacuum pump and remove the needle attached to it that was inserted in the now dried round bottom flask. Insert the needle connected to the argon balloon through the septum sealing the round bottom flask.
    1. Add 4-hydroxymethylphenol ester of 2-acetyloxybenzoic acid (100 mg, 0.349 mmol), 4-dimethylaminopyridine (4 mg, 0.033 mmol) and triethylamine (53 mg, 0.523 mmol) to a separate flask, then dissolved them in anhydrous tetrahydrofuran (5 ml). With a plastic syringe attached to a needle, add this solution to the sealed round bottom flask. Cool the mixture down to 0 °C by placing the flask in an ice water bath.
7. To the previous mixture, add a solution of ethyl fumaroyl chloride (68 mg, 0.419 mmol) in anhydrous tetrahydrofuran (5 ml), drop-wise over a period of 10 min. Stir the resulting solution at 0-5 °C for 2-4 hr.
8. Extract the reaction mixture with ethyl acetate (100 ml) and brine (50 ml).
NOTE: Brine is a saturated solution of sodium chloride (NaCl). To prepare it, place 200 ml of water in a clean glass container then start adding NaCl (while stirring) until it doesn't dissolve anymore.
    1. After extraction, remove the aqueous phase and repeat the latter step two more times.
    2. Dry the mixture by adding sodium sulfate to the organic phase, until the solid does not clump up when the glassware is stirred. Using another Buchner funnel with a fritted disk, filter the mixture to a round bottom flask to remove the sodium sulfate, then evaporate to dryness using a rotary evaporator with the temperature set at 40 °C.
9. Using a TLC, determine a proper mobile phase to use in column chromatography.
NOTE: For this experiment a gradient of 20:80 AcOEt/hexane was used.
10. Prepare a column with silica gel and the appropriate solvent phase. Once the compound has been added, add a layer of sodium sulfate (or sand) to protect the column as the solvent is added.
    1. As the column runs, monitor the eluate by TLC. Spot every other test tube as they are collected from the column. When the product of interest elutes, mix all samples containing pure product into a large round bottom flask, and dry them using a rotary evaporator with the bath temperature set at 40 °C.
    NOTE: Product should appear as a white solid.
11. To confirm the structure of GTCpFE, collect proton and carbon (^{1}H and ^{13}C, respectively) nuclear magnetic resonance (NMR) spectra as per manufacturer's instructions.
NOTE: In this study a 400 MHz FT NMR spectrometer was used to collect the ^{1}H and ^{13}C spectra. Run at least 25 scans at room temperature to obtain enough data resolution. The NMR peaks observed were the following, ^{1}H NMR ($CDCl_3$): δ 1.29 (t, 3 H, 3J = 7.0 Hz, CH_3); 2.31 (s, 3 H, CH_3); 4.26 (q, 2 H, 3J = 7.0 Hz, $COOCH_2$); 5.25 (s, 2 H, OCH_2); 6.89 (s, 2 H, HC=CH); 7.19 (m, 3 H, Ar); 7.42 (m, 3 H, Ar); 7.65 (m, 1 H, Ar); 8.22 (dd, 1 H, 3J = 7.8, 4J = 1.2 Hz, Ar). ^{13}C NMR ($CDCl_3$) δ: 169.70, 164.86, 164.75, 162.89, 151.28, 150.69, 134.76, 134.32, 133.26, 133.16, 132.25, 129.81, 126.26, 124.11, 122.47, 122.04, 66.40, 61.43, 21.05, 14.15.
12. In addition, collect a high resolution mass spectrum using liquid chromatography mass spectrometry in combination with ion trap and time-of flight technology (LCMS-IT-TOF) for accurate mass measurements as per manufacturer's instructions.
NOTE: In this study ($M+NH_4^+$) calculated: 430.1496; observed: 430.1477.

2. GTCPFE Inhibits the NFκB Activity in Breast Cancer Cells

1. **Cell Culture Conditions**
    1. Maintain human breast cancer cell lines, MCF-7 and MDA-MB-231 as per standard cell culture techniques and propagate in a humidified incubator at 37 °C with 5% CO_2.
        1. Prepare MCF-7 cell medium from Roswell Park Memorial Institute (RPMI) 1640 medium with phenol red supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 2 mM L-glutamine, 1% antibiotics penicillin-streptomycin, and 6 ng/ml insulin. Prepare MDA-MB-231 cell medium from Improved Minimum Essential Medium (IMEM) supplemented with 5% FBS, 1% non-essential amino acids, 2 mM L-glutamine, and 1% antibiotics penicillin-streptomycin.

2. **NFκB-RE Luciferase Reporter Assay**
    1. Trypsinize breast cancer MCF-7 cells using 0.25% trypsin for 5 min at 37 °C, manually count using a hemocytometer and seed in 24-well plates at 90,000 cells per well density.
    2. The following day, co-transfect cells with plasmid DNA of an NFκB response element (NFκB-RE) luciferase construct, 1 µg/well, along with the promoterless Renilla luciferase construct, 0.2 µg/well. Perform transfections for each treatment group in triplicate.
        1. Wash cells twice with PBS, and incubate with mixtures of plasmid DNA and 1 µl per well of transfection reagent (*e.g.* lipofectamine) in serum free media. After 6 hr, change the medium to 1 ml of regular medium (without the antibiotics penicillin and streptomycin).
    3. After 16 hr, add 1 µl of vehicle or different stock solutions of the GTCpFE or ASA drugs to each well. Dissolve drug stock solutions (100, 50, 20, 10 and 1 mM) in dimethyl sulfoxide at 1,000x concentration. After adding the drugs, incubate cells for 2 hr at 37 °C.
    4. To activate the NFκB pathway, add the pro-inflammatory cytokine TNFα (10 µg/ml stock solution) into each well for a final concentration of 10 ng/ml and incubate for 4 hr. Include a TNFα alone control. Aspirate the medium and store the cells at -80 °C.
    5. Measure luciferase using a luciferase reporter assay system according to manufacturer's instructions.
    NOTE: When using the luciferase assay system (*e.g.* Dual Luciferase assay system) for the first time, prepare a solution of luciferase assay reagent by re-suspending it in 10 ml of buffer and storing it at -80 °C in 1 ml aliquots.
        1. Prepare 1x lysis buffer by diluting 5x stock buffer with water. Lyse cells in each well using 100 µl 1x lysis buffer. Incubate the plates on an orbital shaker for 15 min, at medium speed.
        2. Label one 1.5 ml tube per sample. Thaw luciferase assay reagent to room temperature (50 µl per sample will be needed) and keep in foil. Prepare 1x quenching reagent in a glass vial, 1:49 dilution in buffer and vortex it (50 µl per sample will be needed).
        3. Add 10 µl of cell lysate to a labeled microcentrifuge tube. Then add 50 µl of luciferase assay reagent and gently vortex. Immediately after, place the tube in a holder and measure the luminescence via the dual luciferase program in the luminometer. Select and press the following: Run Promega Protocols → Dual Glo → OK.
        4. Add 50 µl of the quenching reagent to the test tube and gently vortex. Measure the luminescence. Repeat these steps for each sample.
        5. Analyze data using spreadsheet by normalizing NFκB-RE to the Renilla internal control (NFκB-RE/Renilla). Compare inhibitor data to TNFα only control (TNFα only is set at 100%).

3. **NFκB-Target Gene Transcription**
    1. Seed MCF-7 cells in 6-well plates at 250,000 cells per well in 2 ml medium volume prepared as described in section 2.1.
    2. The following day, add 2 µl of different GTCpFE 1,000x stocks (100, 50, 20, 10 and 1 mM) for 2 hr prior to adding TNFα 10ng/ml for another 2 hr. Run every treatment in triplicate. Include vehicle and TNFα alone controls.
    3. Isolate RNA using the guanidinium thiocyanate-phenol-chloroform extraction method according to the instructions of the manufacturer[22]. Determine RNA concentration (in diethylpyrocarbonate (DEPC)-treated water) and RNA purity using a spectrophotometer. Keep RNA samples on ice or store at -80 °C. Use only RNase-free barrier tips when handling RNA.
    4. Reverse transcribe 0.5 µg total RNA using a commercially available kit of Moloney murine leukemia virus (M-MLV) reverse transcriptase.
    NOTE: The kit includes 5x buffer and dithiothreitol (DTT), together with dNTP mixture, and random hexamers mixture.
        1. Add 0.5 µg of RNA to 200 units of M-MLV, 0.5 mM dNTPs, 100 ng of random hexamers, 10 mM DTT, 1x buffer and DEPC water to a final 10 µl reaction volume. Carry out the reverse transcriptase reaction using a PCR cycler for 50 min at 37 °C, and then 15 min at 70 °C to heat-inactivate the enzyme.
    5. Dilute the resulting cDNA product to 100 µl with double-distilled water and use 2 µl for each subsequent quantitative polymerase chain reaction (PCR) reaction.
        1. Mix forward and reverse primers for the gene of interest at 1.25 µM concentration each. Prepare a master mix with 2 µl double distilled water, 1 µl of 1.25 µM primer mix and 5 µl of 2x of the dye to enable detection of the double-stranded DNA. Load the 2 µl of cDNA and 8 µl of master mix into 96-well PCR plates.
    6. Carry out quantitative PCR using a real time PCR system (40 cycles, 95-60 °C) according to manufacturer's instructions and manually analyze the data.
    NOTE: All PCR primers used have been validated and reported previously[23].
    7. Calculate gene expression fold change using spreadsheet via the ΔΔCt method, with ribosomal protein 36B4 mRNA serving as the internal control[23].

3. GTCpFE Inhibits Breast Cancer Stem Cells *In Vitro*

1. **Mammosphere Formation Assay**

1. Prepare mammosphere (MS) medium by supplementing Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) phenol red-free medium with 1% methyl cellulose. Allow to dissolve by gentle shaking overnight at 4 °C. Filter sterilize the medium and supplement with B27 1x, 1% penicillin and streptomycin, 5 µg/ml insulin, 1 µg/ml hydrocortisone, and 20 ng/ml recombinant human epidermal growth factor.
2. Prepare single cells of MDA-MB-231 cell line by trypsin digestion (trypsin 0.25% for 5 min at 37 °C) of monolayer cultures and filter through mesh sieves. Manually count single dissociated cells and plate in 96-well ultra-low attachment plates at a density of 400 cells/well. The next day, add different concentrations of GTCpFE (*e.g.*, final GTCpFE concentrations: 1, 10, 20, 50 µM) to a final volume is 100 µl. Conduct every treatment in triplicate.
3. After 7 days of culture in the incubator, acquire images via an imaging software using an inverted microscope. Manually count the number MS >75 µm in diameter. Compare the drug treatment to vehicle control.
   NOTE: The diameter of MS >75 µm is determined using the imaging software.

2. **$CD44^+CD24^-$ CSC Immunophenotype**
   1. Trypsinize MDA-MB-231 cells with 0.25% trypsin for 5 min at 37 °C. Count using a hemocytometer and seed in 10 cm dishes at 3 million cells per dish in 10 ml medium as described in section 2.1. Add vehicle (10 µl dimethyl sulfoxide) or GTCpFE (10 µl of 50 mM stock) to cells for 72 hr.
   2. For vehicle or GTCpFE treatment groups, trypsinize cells (as in step 3.2.1) and distribute 1 million cells in 'Test' or 'Control' 5 ml polystyrene tubes containing 2 ml of 1x Hank's Balanced Salt Solution (HBSS) buffer supplemented with 2% FBS.
   3. To stain for the surface markers CD44 and CD24, spin cells down and add 20 µl of each conjugated antibody and HBSS + 2% FBS to Test tubes for a final 100 µl overall volume (1:5 dilution). Add 100 µl of HBSS + 2% FBS to Control tubes. Include CD44-APC conjugated antibody and CD24-PE antibody single stain controls or the IgG immunoisotype controls.
   4. Incubate cells in the dark at 4 °C for 30 min. Spin down cells for 5 min at 400 x g and reconstitute in 200 µl HBSS buffer with 2% FBS. Keep cells on ice in the dark.
   5. Perform Fluorescence-activated cell sorting (FACS) of live cells using a FACS analyzer instrument according to manufacturer's instructions. Collect at least 50,000 events for each tube. Run treatments in triplicate.
      NOTE: Gating is based on controls from no staining (Control tube), IgG immunoisotypes, and the CD44-APC and CD24-PE single stains.
   6. Analyze data using an available flow cytometry software.
      NOTE: The percent of GTCpFE-treated cell with the $CD44^+CD24^-$ immunophenotype is estimated and compared to vehicle control.

Representative Results

In **Figure 1**, the chemical structure of aspirin-fumarate prodrug, GTCpFE, and its inhibitory activity on cytokine induced NFκB pathway in breast cancer cells are indicated. GTCpFE inhibits both NFκB endpoints, NFκB-RE luciferase activity (**Figure 1B**) and expression of NFκB target genes, such as Intercellular Adhesion Molecule 1 (ICAM1), Chemokine C-C Motif Ligand 2 (CCL2), and Tumor Necrosis Factor (TNF) (**Figure 1C**) in MCF-7 breast cancer cells. Calculated inhibitory concentration at 50% (IC_{50}) value on both endpoints is ~20 µM. IC_{50} value is calculated using a graphing software. By comparison ASA itself even at 200 µM (10x IC_{50} of GTCpFE) shows no inhibitory activity (**Figure 1B**, blue line) in breast cancer cells. This indicates that the prodrug strategy of adding fumarate pharmacophore to ASA significantly improves its anti-NFκB activity.

To measure the anti-CSC activity, we used two assays: the mammosphere (MS) formation assay and the population of cells expressing the $CD44^+CD24^-$ immunophenotype, a *bona fide* CSC surface marker in breast cancer[24]. GTCpFE inhibits MS formation of MDA-MB-231 breast cancer cells in a dose dependent manner shown in **Figure 2A**. Similar to NFκB pathway inhibition in adherent cultures, the IC_{50} value for mammosphere formation is ~20 µM. This consistency is expected, given that CSCs rely on NFκB signaling for survival and propagation[16-21]. In addition, GTCpFE pre-treatment resulted in a significant depletion of the $CD44^+CD24^-$ population (**Figure 2B**) in MDA-MB-231 cells. Together, these results establish GTCpFE's ability to effectively target breast CSCs.

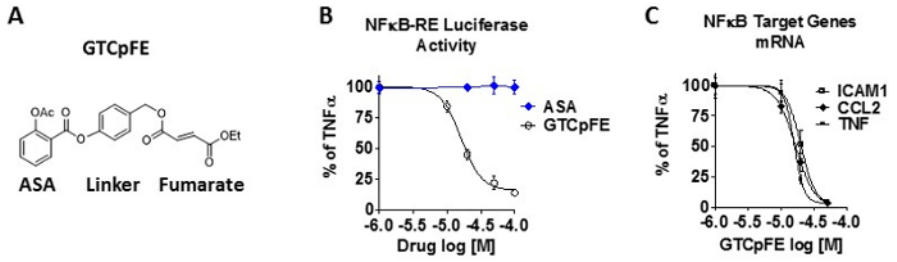

Figure 1: GTCpFE inhibits the NFκB pathway in breast cancer cells. (**A**) The chemical structure of the aspirin-fumarate prodrug, GTCpFE. (**B**-**C**) MCF-7 cells were pre-treated for 2 hr with various concentrations of GTCpFE, ASA, or vehicle followed by treatment with TNFα (10 ng/ml) for 2-4 hr. (**B**) NFκB-RE activity was measured by dual luciferase reporter assay. (**C**) Expression of NFκB target genes, ICAM1, CCL2 and TNF was measured by RT-QPCR. Drug inhibitory activity is plotted as % of TNFα alone. Data are presented as mean ± SEM. This figure has been modified from reference[15]. Please click here to view a larger version of this figure.

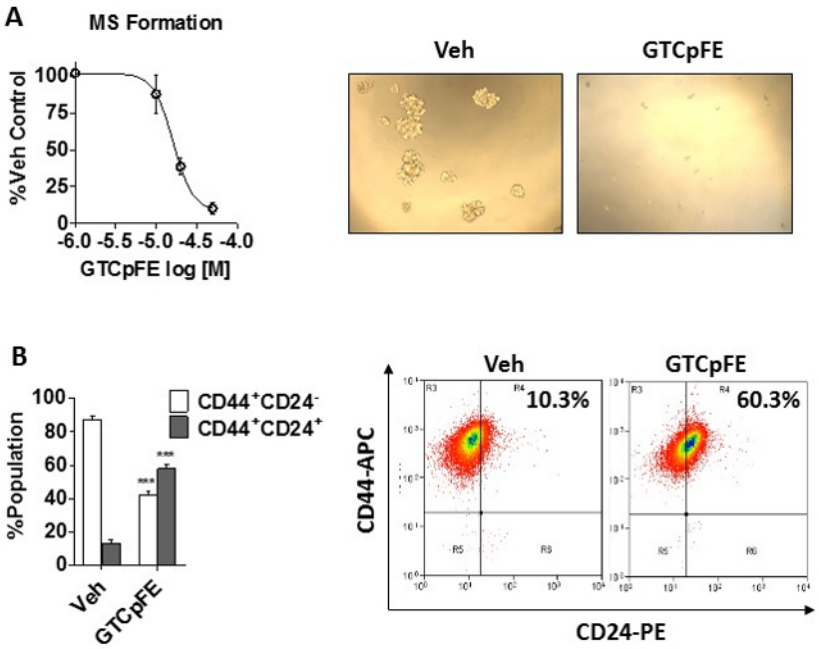

Figure 2: GTCpFE inhibits the mammosphere formation and the $CD44^+CD24^-$ immunophenotype in breast cancer cells. (**A**) Mammosphere (MS) formation of MDA-MB-231 cells was measured after treatment with varying concentrations of GTCpFE. Quantitation of MS growth (left) and representative pictures of MS at 20X (right) are shown. The effect of GTCpFE is plotted as % vehicle control. (**B**) The $CD44^+CD24^-$ population was determined by FACS analysis of MDA-MB-231 cells treated with 50 µM GTCpFE for 72 hr. Quantitation of each population percentage (left) and representative scatter plots from FACS (right) are shown. Data are presented as mean ± SEM and statistical analysis of 2-way ANOVA followed by Tukey posttest. *** P<0.001. This figure has been modified from reference[15].

Discussion

In this protocol, we demonstrated the synthesis of an ASA prodrug, GTCpFE, where the fumarate pharmacophore was incorporated to improve the anti-NFκB activity in breast cancer cells. GTCpFE is an effective NFκB inhibitor, whereas ASA itself is not, even at much higher concentrations. The fumarate moiety has anti-inflammatory properties as shown by its ability to inhibit NFκB signaling in a variety of cell lines and tissues[14,25-29]. The prodrug strategy described herein, is amendable to other malignancies where multiple inflammatory pathways are active and contribute to the pathology. Therefore, GTCpFE may be the prototype for developing new fumarate-based anti-inflammatory and anti-CSC class of drugs. Besides ASA, other non-steroidal anti-inflammatory drugs may also be used. In this case, the synthetic route should be re-designed as well. Ensuring that the new prodrug maintains its anti-NFκB and anti-cyclooxygenase activity via the assays describe herein is critical.

GTCpFE abrogated MS formation and inhibited the $CD44^+CD24^-$ immunophenotype. Together these findings suggest that GTCpFE may also be a promising, clinically relevant anti-inflammatory molecule for eradicating breast CSCs by exploiting CSC's reliance on multiple inflammatory pathways, including the NFκB pathway and the cyclooxygenase 2-prostaglandin E2 axis[16-21,30]. Targeting breast CSCs is important because they are thought to contribute to therapy resistance, recurrence and metastasis[31-34].

The anti-CSCs properties described here, the mammosphere formation and the $CD44^+CD24^-$ immunophenotype, are based on typical *in vitro* assays to assess effect on stemness. Mammosphere formation is a functional assay because it exploits the unique property of stem-like/progenitor cells to survive and grow in serum-free suspension, while more differentiated cells undergo anoikis and die in these conditions[35,36]. However, because a reduction in mammosphere formation may represent (i) increased apoptosis, (ii) decreased proliferation of early progenitor cells or stem cells, (iii) a reduction in stem cell self-renewal, or (iv) interference with anchorage-independent growth, it is best used in conjunction with other anti-CSC assays. The choice of the well-established breast CSC $CD44^+CD24^-$ immunophenotype is based on the pioneering study by Clarke and colleagues who used breast cancer xenografts to isolate a population of cells capable of initiating tumors in immunodeficient mice[24]. Similar to the $CD44^+CD24^-$ immunophenotype, expression of other cell surface markers can be used to isolate stem cells via flow cytometry, but the choice of marker can greatly vary depending on tissues or species (human vs murine model). Alternative assays to measure effects on breast CSCs *in vitro* are (i) the side population technique, which is based on the ability of stem cells to exclude vital dyes via transmembrane transporters[37], and (ii) the ALDEFLUOR assay, which is based on the enzymatic activity of aldehyde dehydrogenase 1 (ALDH1)[38]. Lastly, a suppression of pluripotency genes, transcription factors, or key stemness pathways may also be indicative of anti-CSCs effects. Promising *in vitro* anti-CSC properties should be followed up by *in vivo* characterization. The "gold standard" for assaying anti-CSC properties is *in vivo* tumorigenicity, wherein the ability of drug-treated cells to initiate or seed a xenograft tumor is examined[39,40].

Disclosures

The authors have nothing to disclose.

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