

Substituted Tetrahydroisoquinolines as Microtubule-destabilizing Agents in Triple Negative Human Breast Cancer Cells

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Abstract. Triple-negative breast cancer (TNBC) occurs at greater frequency amongst African-Americans, being characterized by the absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal receptor 2 (HER2). TNBC is often invasive and typically treated with cytostatic agents such as taxanes in combination with anthracyclines or platinum-based drugs. In this study, we synthesized a number of tetrahydroisoquinoline moieties by *N*-amination of substituted isoquinolines by *O*-mesytelene sulfonylhydroxylamine followed by ylide formation and reduction, which yielded the desired, substituted tetrahydroisoquinolines (THIQs) in moderate to good yield. Using a differential scatter plot to identify potential selective ER-modulating drugs in ER-positive control cells (MCF-7) driven by estradiol vs. TNBC (MDA-MB-231) cells, the *in vitro* data showed an absence of effects on the ER (compared to 4-hydroxy-tamoxifen and raloxifene). In contrast, two lead compounds halted proliferation (cytostatic) in MDA-MB-231 TNBC cells at a potency level below 2.5 μ M concomitant with mitotic arrest, attenuated replicative DNA synthesis, halted microtubule nucleation/stunted tubulin polymerization, abnormal expansive cytoskeletal tubulin and actin morphologies with multinucleation of cells. The most effective cytostatic compounds GM-4-53 and GM-3-121 blocked replicative processes at the G₂ growth phase. These findings suggest that specific THIQs work independently of the ER, by holding static the microtubule network thereby preventing mitosis. Future work is required to establish the safety

and efficacy of these drugs and their potential adjunct therapeutic gain in the presence of taxanes in TNBC.

Triple-negative breast cancer (TNBC) is highly invasive and associated with greater rates of mortality in African-Americans (1). TNBC is typically associated with the breast cancer type 1 susceptibility protein (*BRCA1*), *p53* mutation, characterized by the lack of estrogen receptor (ER), progesterone (PR) receptor and human epidermal growth factor receptor 2 (HER2) (2-4). Treating this type of malignancy excludes application of hormone-based drugs, rendering heavy reliance on taxane-based drugs, such as paclitaxel with combined treatment of anthracyclines (5) or subsequent platinum-based therapies (6). Paclitaxel mediates its effects through acting as a cytostatic agent, which impairs cell division through paralyzing microtubule dynamics and suppressing detachment from centrosomes. However, its use can bring on multidrug resistance, rendering few available options for terminally ill patients facing aggressive TNBC (7).

Recently, reports have suggested that natural or synthesized tetrahydroisoquinoline derivatives (THIQs) could serve as a potential class of drugs for breast cancer some of which target the overexpression of P-glycoproteins (8, 9), overcome multidrug resistance (10), impair migration and invasion (11), and exert antiproliferative effects (12-14) by inducing cell cycle arrest at G₂/M phase (15). In our recent studies, we reported the synthesis of THIQs by *N*-amination of isoquinoline using hydroxylamine-*O*-sulfonic acid followed by ylide formation and reduction, which yielded the desired, *N*-substituted THIQs (16-18). In this study, we evaluated the effects of these THIQs on growth parameters in TNBC cells.

Materials and Methods

Hanks balanced salt solution (HBSS), [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] (HEPES), ethanol, 24-well plates, 96-well plates, general reagents and supplies were all purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and VWR

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Key Words: Triple-negative breast cancer, taxanes, tetrahydroisoquinolines.

International (Radnor, PA, USA). Imaging probes were supplied by Life Technologies (Grand Island, NY, USA).

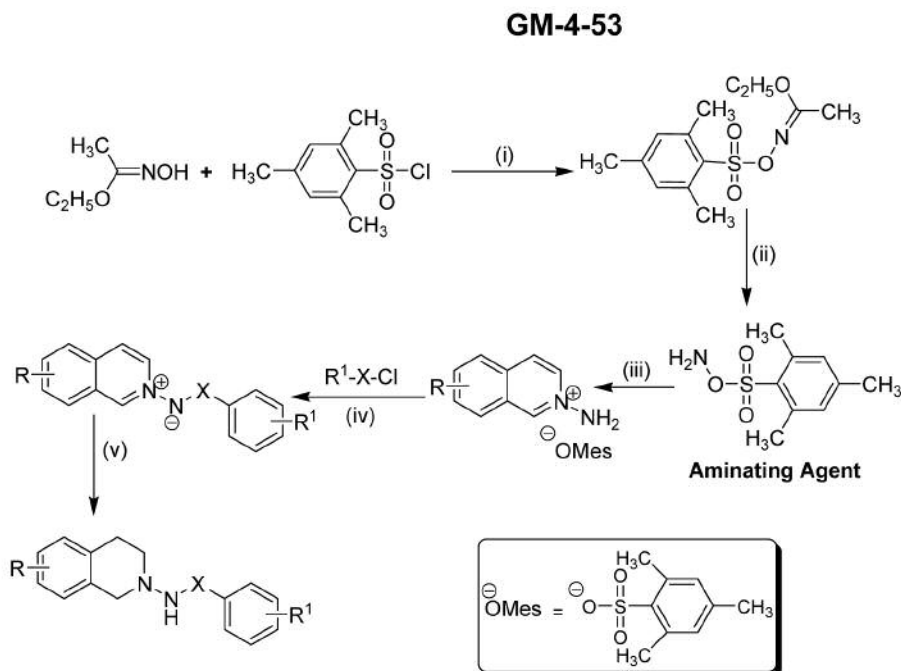
Synthesis. Synthesis of THIQs has been previously described (16-18) for GM-3-121. The synthesis of GM-4-53 was accomplished following the same General procedure as described in our earlier work. *O*-mesitylene sulfonyl hydroxylamine (MSH) (3) was used to prepare the *N*-amino salt as an aminating agent as described (19). An ice cooled solution of 7-hydroxyisoquinoline (2.0 g, 13.78 mmol) in 30 ml of dry methylene chloride, and 15 ml of dry methanol was added drop wise to *O*-mesitylenesulfonylhydroxylamine (2.97 g, 13.78 mmol) in 10 ml of dry methylene chloride over 5 min with stirring. The reaction stirred at 0°C. for 6 h. 70 ml of ether was added and the suspension filtered. The precipitate was recrystallized from ethyl acetate to give 2-amino-7-hydroxyisoquinolinium 2,4,6-trimethylbenzenesulfonate in 60.5% yield. Isolated products were used as such in further reactions.

General procedure for acylation. An ice-cold solution of 2-amino-7-hydroxyisoquinolinium 2,4,6-trimethylbenzenesulfonate (8 mmol) in 25 ml of anhydrous tetrahydrofuran (THF) containing triethylamine, was added to a 4-ethylbenzoyl chloride (12 mmol) with stirring. The reaction was allowed to proceed for 12 h at 70°C. After cooling to room temperature, the reaction was quenched by adding 25 ml of saturated aqueous sodium bicarbonate solution. The mixture was repeatedly shaken in a separation funnel and allowed to stand for few minutes. Extraction with dichloromethane (2x100 ml), and drying over anhydrous sodium sulfate, and removal of the solvent *in vacuo* gave the crude product that was purified by combiflash chromatography

using ethyl acetate:dichloromethane (3:2 v/v) as an eluent. The ylide was obtained as light yellow solid in 50% yield. ¹HNMR (CDCl₃) δ (ppm): 1.26 (t, 3H, J=7.5 Hz, -CH₂-CH₃), 2.66-2.75 (q, 2H, J=8.1, 7.8 Hz, -CH₂-CH₃), 7.28 (d, 2H, J=8.7 Hz, C₃, C₅-H), 7.39 (d, 1H, J=2.1Hz, C₈-H), 7.84 (d, 1H, J=5.7 Hz, C₆-H), 7.99 (d, 1H, J=2.1 Hz, C₅-H), 8.05 (d, 2H, J=8.1 Hz, C₄-H), 8.17 (d, 2H, J=8.4 Hz, C₂, C₆-H), 8.51 (d, 1H, J=6.0 Hz, C₃-H), 9.9 (s, 1H, C₁-H).

General procedure for reduction. A solution of ylide (5 mmol) in 20 ml of absolute ethanol was added drop-wise to a solution of sodium borohydride (50 mmol) in 25 ml of absolute ethanol pre-cooled to 0°C. The reaction was allowed to proceed for 5-7 h at 0°C with stirring. Water (35 ml) was added, and allowed to warm up to room temperature. Extraction with dichloromethane (3x50 ml), drying over anhydrous sodium sulfate, and removal of the solvent *in vacuo* gave the crude product, which was purified by combiflash chromatography using ethyl acetate:hexane (2:3 v/v) as an eluent to afford pure 4-ethyl-*N*-(7-hydroxy-3,4-dihydroisoquinolin-2-(1*H*)-yl)benzamide (GM-4-53) as a white solid in 65.0% yield. ¹HNMR (CDCl₃) δ (ppm): 1.18 (t, 3H, J=7.5 Hz, -CH₂-CH₃), 2.64-2.72 (q, 2H, J=7.5 Hz, -CH₂-CH₃), 2.86 (t, 2H, J=6.0 Hz, C₄-H), 3.18 (t, 2H, J=5.7 Hz, C₃-H), 3.94 (s, 2H, C₁-H), 6.35 (d, 1H, J=2.1, 1.8 Hz, C₈-H), 6.58 (dd, 1H, J=2.7, 5.4 Hz, C₆-H), 6.79 (d, 1H, J=8.4 Hz, C₅-H), 7.09 (s, 1H, -NH, D₂O exchange), 7.17 (d, 2H, J=8.1 Hz, C₃, C₅-H), 7.65 (d, 2H, J=8.1 Hz, C₂, C₆-H).

Cell culture. MDA-MB-231 (ATCC® HTB-26™) human breast cancer cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). MDA-MB-231 cells were



Reaction Conditions: (i) DMF, 0 °C, 45 min, (ii) 70 % HClO₄, *p*-dioxane, 0 °C, 45 min, (iii) Substituted isoquinoline, dry CH₂Cl₂: dry MeOH (2:1), 0 °C, 5h, (iv) 4-substituted acyl/sulfonyl chlorides, dry THF, 70 °C, 12h, (v) NaBH₄, abs. EtOH, 7 h; R = OH, Br; X = CO, SO₂; R¹ = 4-C₂H₅, 4-*tert*-C₄H₉, 4-OCH₃, 2-OCH₃, 2-C₂H₅; X = CO, SO₂

cultured in ATCC-formulated Leibowitz's L-15 medium, supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/0.1 mg/ml). After confluence, the cells were sub-cultured and grown in Dulbecco's modified Eagle's medium (DMEM) containing phenol red, 10% FBS, 4 mM L-glutamine, 20 μ M sodium pyruvate and penicillin/streptomycin (100 U/0.1 mg/ml). Cells were maintained at 37°C in 5% CO₂/atmosphere and every 2-5 days, the medium was replaced and cells sub-cultured. For experiments, plating medium consisted of DMEM with 1% (for determination of cell-cycle phase) or 5% (for proliferation studies) FBS plus penicillin/streptomycin (100 U/0.1 mg/ml), 25 mM glucose, 2 mM sodium pyruvate and 3 mM L-glutamine.

Proliferation studies. Compounds were dissolved in DMSO, vortexed and stored at -20°C in the dark. A stock solution for all experimental compounds were prepared in HBSS plus 5 mM HEPES, adjusted to a pH of 7.4. Briefly, 96-well plates contained cells at a low cell plating density (0.04×10⁶/well) to which compounds of equal concentration were added and cell proliferation was evaluated after 72 h. Compounds were assessed for influence on cell count, ranked for potency, and 50% inhibitory concentration (IC₅₀) values were calculated by regression analysis.

Viable cell count. Viable cells were quantified using resazurin (Alamar Blue) indicator dye. A working solution of resazurin was prepared in sterile phosphate-buffered saline (PBS)-phenol red (0.5 mg/ml) and added (15% v/v) to each sample in a 96-well plate. Samples were returned to the incubator for 2-4 h, and reduction of the dye (to resorufin, a fluorescent compound) by viable cells was quantitatively analyzed using a Synergy HTX multi-mode reader (Bio-Tek, Winooski, VT, USA) with excitation/emission wavelength settings at 550/580.

[³H] Thymidine incorporation (TdR). Replicative DNA synthesis was assessed by radiolabeled incorporation of TdR. Briefly, cells were plated in 24-well plates, containing HBSS (control) or test compound then supplemented with 1.5 μ Ci/ml TdR. After a 72-h incubation period, the cells were scraped, centrifuged, pelleted and washed four times with PBS. A final rinse was achieved by cell redistribution in PBS and filtering through a 0.2 μ m microcentrifuge filter which was then placed in scintillation cocktail and vortexed for 30 sec. [³H] Radioactivity was determined using a Beckman LS 6500 liquid scintillation counter (Beckman-Coulter Inc, Indianapolis IN, USA).

Tubulin polymerization. Tubulin polymerization assay (Cytoskeleton, Inc., Denver, CO, USA) was used for determining the effects of drugs. The assay utilizes neuronal tubulin which creates a polymerization curve showing three stages of microtubule formation: nucleation, growth, and equilibrium, which were measured at over a 60-min period and maintained at a temperature of 40°C. The data were acquired with a Synergy HTX multi-mode reader (Bio-Tek, Winooski, VT, USA) with excitation/emission wavelength settings at 340/460.

Cell imaging. Alexa Fluor® 488 Phalloidin and propidium iodide (PI) fluorescent dyes were used to corroborate proliferation indirectly by DNA/cytoskeleton F-actin. Briefly, stock solutions containing fluorescent probes were prepared by dissolving 5 mg/ml ethanol, which were then subsequently diluted in HBSS and

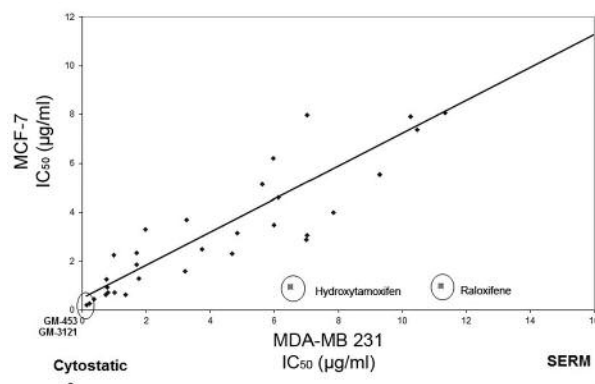


Figure 1. Correlation scatterplot to identify potential selective estrogen receptor modulators (SERM) drugs by evaluation of the antiproliferative effects in estrogen receptor-positive (ER⁺) cells (MCF 7) driven by estradiol vs. triple-negative (MDA-MB-231) cells. The data represent 50% inhibitory concentration (IC₅₀) of each compound against each cell line.

added to cells: final dye concentration: 5 μ g/ml PI and 6.6 μ M phalloidin. Photographic images for tubulin were acquired using a TubulinTracker™ Oregon Green® 488 Taxol, bis-acetate probe. Samples were analyzed photographically using a fluorescent/inverted microscope, CCD camera and data acquisition using ToupTek View (TouTek Photonics Co, Zhejiang, P.R China).

Determination of cell-cycle phase. Cells were plated in 24-well plates at 0.2×10⁶ cells/ml and cultured in low-serum medium for 24 h to synchronize the cell cycle. After 24 hours, the low-serum medium was removed, and DMEM with 5% FBS and 25 mM glucose was added before treatment with test compounds cultured alongside both positive untreated controls and negative controls containing paclitaxel (5 μ M). After 24 h, cells were trypsinized, centrifuged and washed twice with assay buffer (Cayman Chemical, Ann Arbor, MI, USA), re-suspended to a density of 10⁶ cells/ml in a cell suspension, fixed and stored at -20°C. After 48 h, fixative was removed, and the pellet was placed in 0.5 ml of a staining solution containing PI and RNase. A solution The distribution of DNA in all cell-cycle phases was assayed by flow cytometry in replicates. The proportion of cells in each stage was assayed within 2 h by using an FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). In each sample, a total of 10,000 individual events from the gated subpopulation were analyzed separately. CellQuest software (BD Biosciences, San Jose, CA, USA) was used for acquisition and analysis of the data, and the percentage of cells in each phase was determined by using ModFit LT 3.0 software (Verity Software House, Topsham, ME, USA).

Data analysis. Statistical analysis was performed using Graph Pad Prism (version 3.0; Graph Pad Software Inc. San Diego, CA, USA) with the significance of the difference between groups assessed using a one-way ANOVA, followed by Tukey *post hoc* means comparison test or Student's *t*-test. IC₅₀s were determined by regression analysis using Origin Software (OriginLab, Northampton, MA, USA).

Table I. Growth-inhibitory properties of derivatives on MDA-MB-231 human breast carcinoma cells. The data represent 72-h growth-inhibitory potency established by 50% inhibitory concentration (IC_{50}) calculated from regression analysis of a minimum of six concentrations between 169 nM-30 μ M, n=4. Lethal concentrations 50 (LC_{50} s) were all in excess of 30 μ M, reflecting lack of toxicity at 24 h.

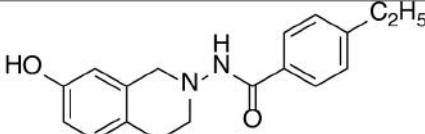
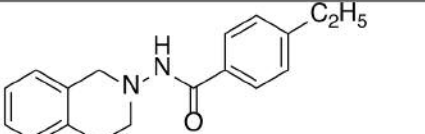
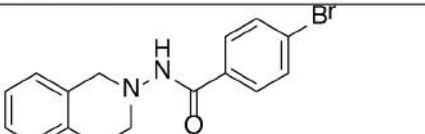
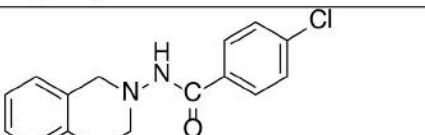
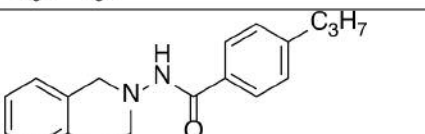
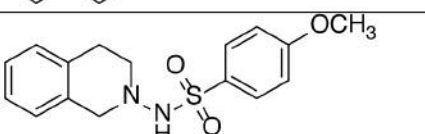
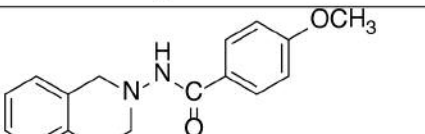
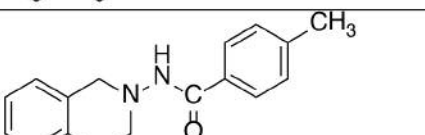
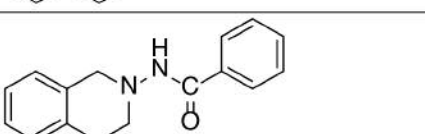
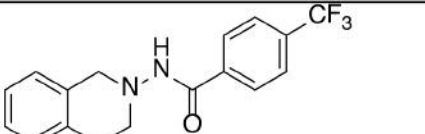
ID	Compound	Structure	IC_{50} (μ M)	LC_{50} (μ M)
	Paclitaxel		<0.169	>30
1	GM-4-53		1.12	>30
2	GM-3-121		2.11	>30
3	GM-3-135		3.56	>30
4	GM-3-18		5.58	>30
5	GM-3-122		6.29	>30
6	GM-3-13		6.78	>30
7	GM-3-16		9.30	>30
8	GM-3-19		>30	>30
9	GM3-15		>30	>30
10	GM-3-143		>30	>30

Table I. Continued

Table I. *Continued*

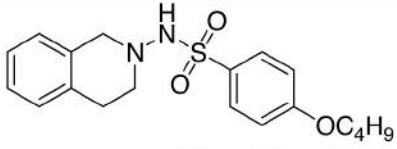
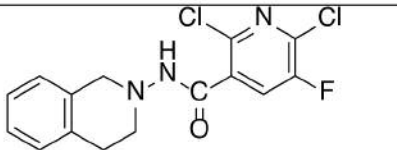
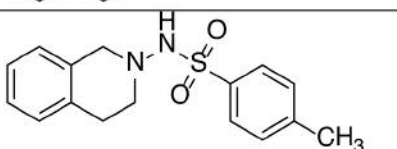
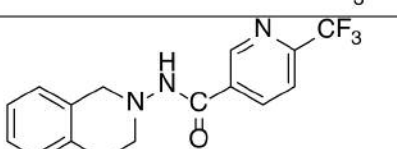
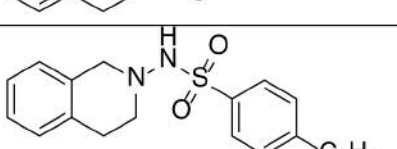
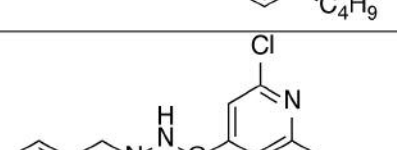
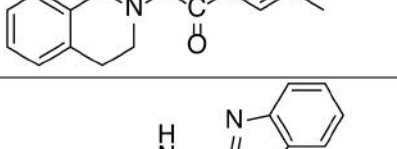
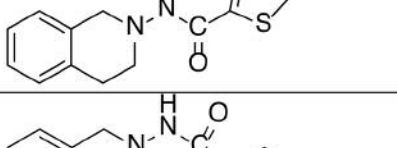
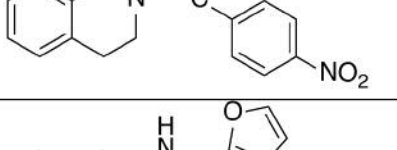
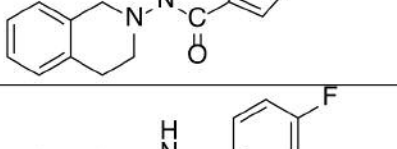
ID	Compound	Structure	IC ₅₀ (μM)	LC ₅₀ (μM)
11	GM-4-5		>30	>30
12	GM-3-180		>30	>30
13	GM-3-146		>30	>30
14	GM-3-178		>30	>30
15	GM-3-162		>30	>30
16	GM-3-174		>30	>30
17	GM-3177		>30	>30
18	GM-3-155		>30	>30
19	GM-3-168		>30	>30
20	GM-3-123		>30	>30

Table I. *Continued*

Table I. Continued

ID	Compound	Structure	IC ₅₀ (μM)	LC ₅₀ (μM)
21	GM-3-144		>30	>30
22	GM-3-148		>30	>30
23	GM-3-150		>30	>30
24	GM-3-172		>30	>30
25	GM-3-156		>30	>30

Results

Antiproliferative effects of synthesized THIQs were initially evaluated by generating a scatterplot with IC₅₀ values for each THIQ against MCF-7 (ER⁺) cells treated with estradiol *versus* those against MDA-MB-231 TNBC cells. A differential becomes evident where selective estrogen receptor modulators (SERM) positive controls (Raloxifene, hydroxytamoxifen) have specificity for halting proliferation in estradiol-treated MCF-7 cells, being relatively weak against TNBC cells (Figure 1). Figure 1 demonstrates a lack of SERM characteristics of any THIQ synthesized, which inhibited growth equally in receptor positive or negative cell lines. All compounds were evaluated for antiproliferative effects, with the IC₅₀ given in Table I and regression analysis for the most potent THIQs GM-4-53 and GM-3-121 in Figure 2. GM-4-53 and GM-3-121 were equally effective in halting proliferation without toxicity.

GM-4-53 and GM-3-121 were further evaluated for effects on replicative DNA synthesis using radio-labeled incorporation of TdR (Figure 3), findings which showed significant losses

that would otherwise be required for cell division. Likewise, these changes correlated with static cytoskeletal changes fostering giant multinucleated cells in a similar fashion often reported for paclitaxel (20, 21) (Figure 4A). These changes occurred tantamount to altered polymerized tubulin (Figure 4B) which is otherwise required to disassemble and reform into spindle platforms for chromosome assembly during cell division. While it is known that paclitaxel basically prevents the de-polymerization of microtubules, how GM-4-53 and GM-3-121 alter polymerization is unknown.

In the next part of the study, we assessed *ex vitro* tubulin polymerization, (Figure 5). The data showed that both THIQs prevented the formation of polymerized tubulin, in contrast to paclitaxel which blocked the de-polymerization process. While these findings corroborate that GM-4-53 and GM-3-121 indeed work as microtubule de-stabilizing agents, effects on the phase of cell cycle halted in mitosis are unknown. We next evaluated interference with the cell cycle compared to paclitaxel using flow cytometry (Figure 6A-B), results which showed comparable mitotic arrest at the G₂ growth phase.

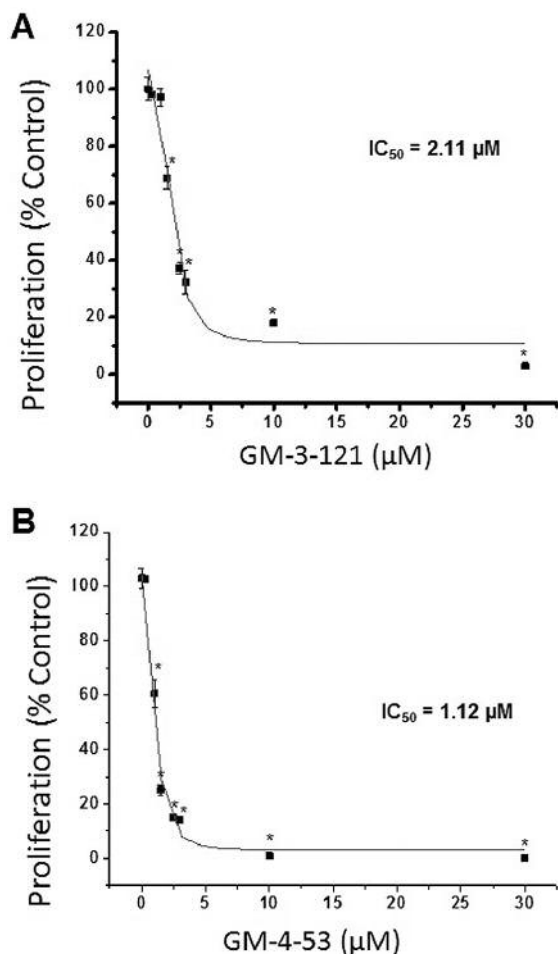


Figure 2. Growth-inhibitory dose-response profiles for lead antiproliferative drugs: GM-4-53 and GM-3-121. The data represent proliferation relative to the control and are presented as the mean \pm S.E.M., $n=4$. * $p<0.05$: Significantly different from the control with a one-way ANOVA followed by a Tukey post hoc test.

Discussion

TNBC is typically defined by the absence of hormone receptor expression (ER, PR and HER2). Lack of hormone receptor expression equals to limited treatment options, due to the lack of any effect of hormonal receptor antagonists (fulvestrant, tamoxifen) or aromatase inhibitors (exemestane, letrozole), and a greater reliance on cytostatic taxane drugs. While taxanes are considered first-line drugs for TNBC, alternative and augmentative adjunct therapies are being explored, including applications using THIQ derivatives. While there is meager research in this particular area, there is an indication that a combination of THIQs with taxanes could overcome multidrug resistance (8, 9) and at the same time augment cytostatic effects of microtubule-binding agents (12-14).

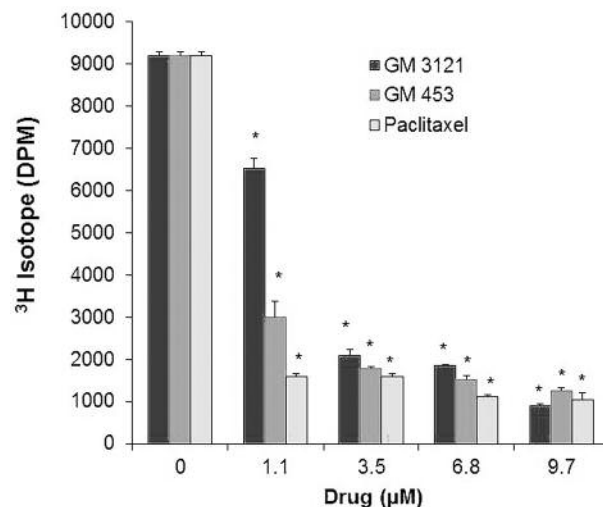


Figure 3. Effect of paclitaxel, GM-4-53 and GM-3-121 on replicative DNA synthesis. The data represent radiolabeled incorporation of [^3H] thymidine (TdR) after 72 h, under various treatments. The data represent disintegrations per minute (DPM) as a percentage of the control and are presented as the mean \pm S.E.M., $n=4$. *Significantly different from the untreated controls at $p<0.05$ using a one-way ANOVA followed by a Tukey post hoc test.

In this study, we synthesized, evaluated and confirmed the efficacy of different THIQ compounds in blocking mitosis, altering cytoskeletal features and arresting cell progression at the G_2 phase relative to paclitaxel in TNBC. These findings show THIQs to be relatively weak in strength compared to paclitaxel, where neither of these mediate effects via antagonizing the ER, evidenced by equal efficacy in estradiol-driven growth in MCF7 cells vs. TNBC MDA-MB-231 cells. On the other hand, while paclitaxel and THIQs both negatively affect polymerization of tubulin, resulting in halt of cell proliferation, the manner in which THIQs target tubulin differs from that of paclitaxel. These findings suggest that THIQs block the polymerization of tubulin, whereas paclitaxel prevents de-polymerization, both of which result in net loss of microtubule assembly. A negative impact on tubulin by either process would then prevent chromosomes from achieving a metaphase spindle configuration, which would then lead to halted mitosis, as found here. Using immunocytochemistry, we demonstrate that both THIQs and paclitaxel are associated with formation of giant multinucleated cells, with grossly abnormal actin and tubulin orientation, a known consequence of mitotic catastrophe and disturbed cytokinesis (21-23).

The correlation of structure and activity indicate the importance of the basic structural characteristics of these cytostatic compounds (Table I), where the substituents at the *para* position, especially the lower alkyl chains such ethyl and

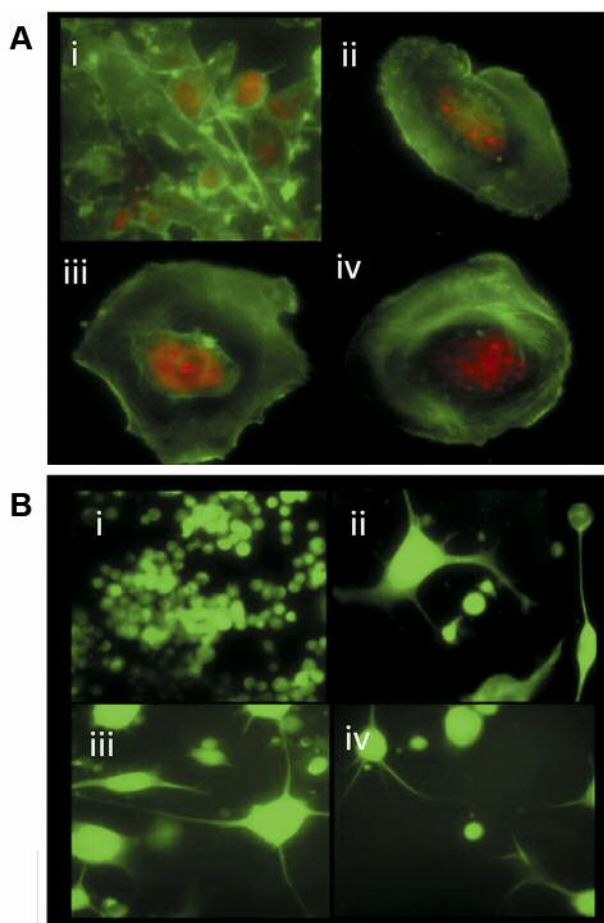
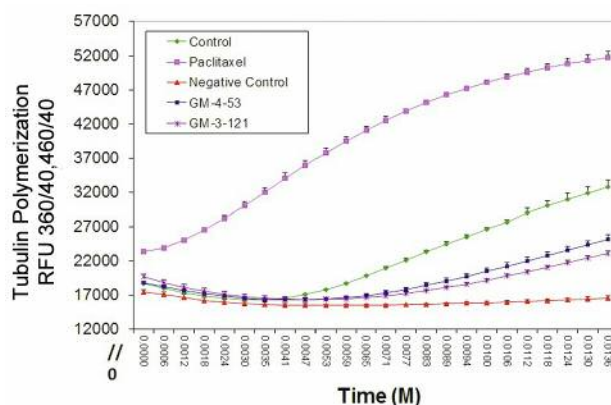


Figure 4. Antiproliferative effects of agents compared with the control in MDA-MB-231 cells. A: Actin cytoskeletal changes shown by fluorescence-stained actin: Alexa Fluor® 488 Phalloidin, with a nuclear counterstain of propidium iodide. B: Polymerized tubulin structure in MDA-MB-231 cells as shown by fluorescence-stained tubulin: Tubulin Tracker™ Green (Oregon Green® 488 Taxol, bis-acetate). i: Control; ii: paclitaxel (5 μ M); iii: GM-4-53 (5 μ M); and iv: GM-3-121(5 μ M).

propyl moieties, and *ortho*, *para* directing substituents such as Br, Cl and OCH₃ on the *N*-substituted phenyl ring contributed to the antiproliferative activity. On the other hand, various *N*-substituted heterocycles, *meta* directing and electronegative substituents, methyl and higher alkyl chains and alkoxides even on the *para* position of the benzene ring or heterocycle did not contribute to the biological activity. Apart from the *para* position, substitutions at all other positions resulted in a decrease in activity. These initial results would guide us in the product optimization studies in the future.

Further work will be required to derivatize the lead structures in order to gain greater potency, to assess molecular targets in detail, and determine if these compounds can affect the tubulin colchicine site, which



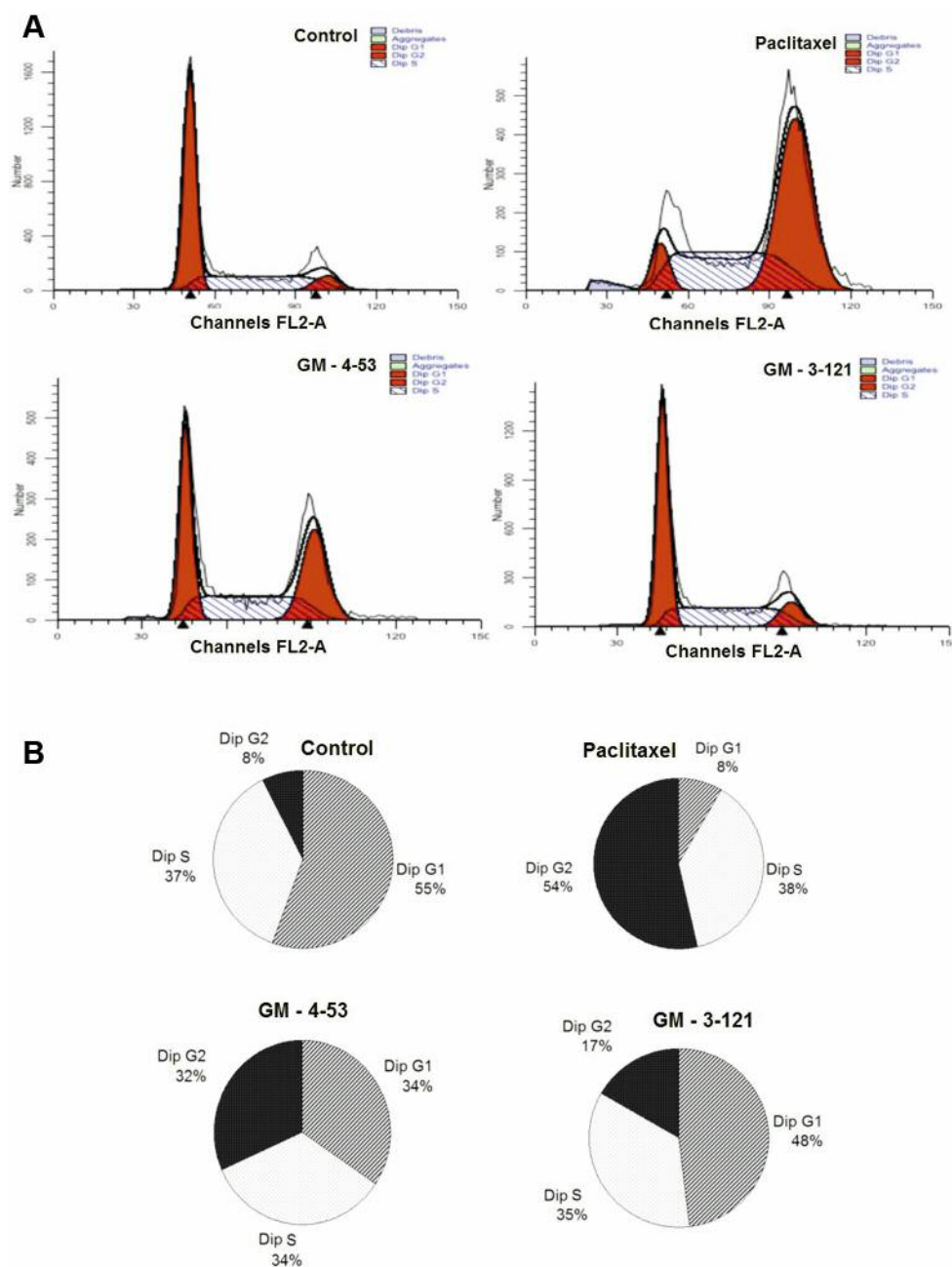


Figure 6. The effect of paclitaxel, GM-3-121, and GM-4-53 all evaluated at 5 μ M vs. the control on the gated population and cell-cycle distribution in MDA-MB 231 cells after cell synchronization and a subsequent 24-h exposure period. The data are expressed as the percentage phase count presented as a histogram (A) and pie chart (B) determined from experimental replicates. Dip, Diploid.

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