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ARTICLE

Design, Synthesis and Evaluation of Cytotoxic Properties of Bisamino Glucosylated Antitumor Ether Lipids against Cancer Cells and Cancer Stem Cells†

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Glycosylated Antitumor Ether Lipids (GAELs) are a class of amphiphilic antitumor agents that kill cancer cells by a non-apoptotic pathway. Previous studies have shown that 2-amino-2-deoxy-D-*gluco*-based GAELs such as **α-GLN** and **β-GLN** show greatly improved antitumor activity against epithelial cancer cells and stem cells. To further optimize the bioactivity, we prepared a series of diamino-D-*gluco*-based GAELs and their analogs, and screened them against a panel of human epithelial cancer cell lines and cancer stem cells. Most of the new GAEL analogs are more potent than chlorambucil, cisplatin and salinomycin. The most potent bisamine-based GAEL analogs **1, 2, 4** and **8** analogs showed 2- to 3-fold enhanced cytotoxicity against various cancer cell lines when compared to **β-GLN**, indicating that the addition of a second amino group enhances the cytotoxic effect. The effect of the most active dicationic GAELs **1** and **4** on cancer stem cell isolated from breast (BT-474) and prostate (DU-145) revealed that the two GAELs inhibited the formation of tumor spheres and resulted in > 95 % loss of viability of the cancer stem cells at 5 µM. Activity of GAEL **1** against BT-474 cancer stem cells is superior to that of salinomycin.

Introduction

Cancer remains a major health problem worldwide despite the huge investment to find effective treatments for the disease. A recent UN report projects that global cancer cases will rise from 14 million to 22 million per year within the next two decades, with annual cancer deaths rising from 8.2 million to 13 million.[1] The major problems impeding the development of cures for cancer are resistance to drugs, resistance to radiotherapy, and metastasis. Many of the existing anticancer drugs act by disrupting cell DNA, preventing DNA synthesis, and/or targeting microtubules, while radiotherapy kills cells by damaging DNA. The cellular perturbation caused by these treatments induces apoptosis which kills the cancer cells. While many drugs are initially successful in killing cancer cells resulting in tumor shrinkage, there is invariably a relapse and the tumor reappears with cells that resist existing chemotherapeutic agents[2,3,4,5] so the tumors are refractory to treatment leading to metastases and death. There are also very few drugs, if any, for treatment of metastasized cancer.

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There is increasing evidence that the relapse of tumors and development of drug and radiation resistant tumors as well as progression to metastases may be due to the presence of a small population of cells in the tumor called cancer stem cells (CSCs) or tumor initiating cells. CSCs are distinct from the cells of the bulk tumor in having the capacity for self-renewal, asymmetric division, and differentiation. CSCs have been identified and isolated from virtually all solid and hematological tumors.[6,7] These cells resist apoptotic cell death induced by chemotherapy and radiotherapy, and may ultimately generate drug-resistant differentiated cells of the recurrent bulk tumor.[6,7] In light of the potential role these cells play in tumor relapse, drug resistance, and metastases, effective cancer treatment will require targeting the CSCs along with the bulk differentiated cells of the tumor.

Only few compounds that kill CSCs have been identified. They include parthenolide, salinomycin, metformin, lapatinib, and mitoVES.[7] The mechanisms via which they achieve this are not known but salinomycin, parthenolide and the biguanide, metformin, induce apoptosis in various human cancer cells.[8-12] The potential of the ionophore antibiotic salinomycin is reinforced by its ability to kill highly multidrug- and apoptosis-resistant cancer cells[11-13] and CSCs.[11,15-17] Antitumor ether lipids (AELs) are synthetic lipids that possess anticancer activity and comprise three subclasses; the alkyllysophospholipid (ALP), alkylphosphocholine (APC) and the non-phosphorylated glycosylated antitumor ether lipids (GAELs). The cytotoxic properties of GAELs, prototypified by **α- GLN** and **β-GLN**, have

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been established to be superior to the most studied AEL, edelfosine (Figure 1).

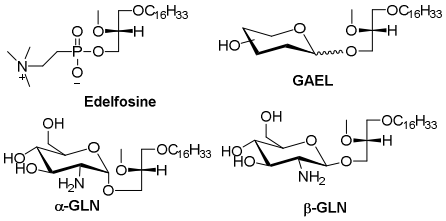


Figure 1 Structures of various glycosylated antitumor ether lipids (GAELs) and edelfosine, an antitumor ether lipid (AEL) of the ALP subclass

Unlike ALPs and APCs which kill cells by apoptosis, GAELs kill cells by an apoptotic-independent mechanism.[18-21] Though the mechanism of action of GAELs is yet to be fully understood, it appears to involve the perturbation of endocytosis pathway to generate large acidic vacuoles that ultimately leads to the release of acid hydrolases to induce cell death.[19-21] This ability to kill cells by an apoptosis-independent pathway led to the postulation that GAELs could kill CSCs because their mode of action will not be impacted by the strategies cells use to prevent death by apoptosis. Recently this hypothesis was validated by the demonstration that a number of GAELs were cytotoxic against CSCs isolated from BT-474 cell lines.[22]

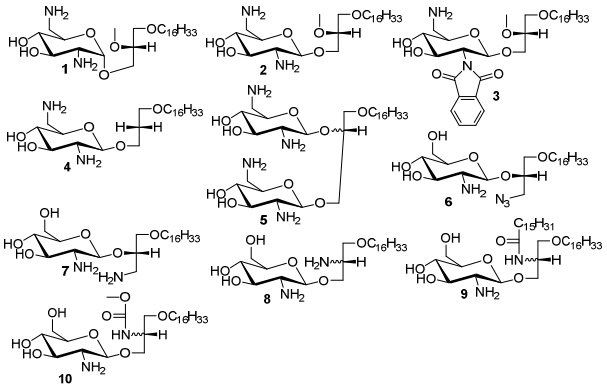
Our ongoing structure activity studies on GAELs have led to the observation that the anticancer potency of GAELs is intimately linked to their cationic nature. Our previously identified leads, **α-GLN** and **β-GLN**, contain a positively charged 2-amino group in the *gluco*-portion of the glycolipid (Figure 1). Replacement of the 2-amino substituent by a neutral hydroxyl or azido group, as well as replacement by a cationic guanidino and secondary amine-based substituents including benzyl amine-substituted groups, resulted in more than 3-fold reduced activity against various cancer cell lines.[23,24] The observation that primary amine-based GAELs are significantly more potent than their non-amine-based counterparts raised the question whether introduction of a second primary amino group in GAELs could further enhance the antitumor properties. We disclose here our structure activity study on diamine-based as well as *N*-substituted *gluco*-based GAEL analogs **1**-**10** (Figure 2).

Results

Chemistry

To study the effects of diamino and N-substituted GAELs, a second amino substituent was introduced at the C_6-position of glucose as shown in compounds **1** and **2** (Figure. 2). Besides the presence of a second amino substituent, glycolipids **1** and **2** were also selected to study the nature of the glycosidic linkage on antitumor activity as previous reports had indicated

Figure 2. Structures of diamine-based and N-substituted



glucosylated antitumor ether lipids (GAELs) used in this study.

that the anomeric configuration can influence the antitumor potency in monocationic GAELs.[18,21,23] Compound **3** with a phthalimido group at the C_2-position of glucose was synthesized to study how the absence of a cationic charge at that position affects cytotoxicity. In addition, the phthalimido group was selected as this group exhibits significant cytotoxicity against both human and murine cancer cell lines.[25,26] Compounds **4** and **5** were synthesized to explore the effect of the methoxy substituent at the *sn*-2 position of the glycero moiety. Previous studies on *gluco*-based GAELs had shown that the nature of the methoxy substituent is not critical for antitumor activity and can be replaced by hydrogen.[23] Another reason for synthesizing tetraamine-containing bisglycolipid **5** was based on the hypothesis that replacement of the methoxy substituent by an additional 2,6-diamino-β-D-glucose moiety may lead to enhanced antitumor activity as seen for other polycationic structures.[27] Dicationic glycolipids **7** and **8** were synthesized to explore how the positioning of a second amino substituent on the glycerol moiety affects the antitumor properties. Azido compound **6** was also prepared to study how the absence of a cationic charge in **7** affects cytotoxicity. Compound **9** was synthesized to explore how the presence of two lipid tails and modification of the free amino substituent at the *sn*-2-position affect the biological properties of compound **8**. We also synthesized carbamate **10** to explore whether the presence of a methoxy carbamate substituent at the *sn*-2-position of the glycerolipid will induce prostate cancer selectivity based on previous findings.[28]

Compounds **5**, **8**, **9**, and **10** were studied as diastereomeric 1:1 mixture based on the stereochemistry at *sn*-2 position of the glycerolipid. We have previously reported that the stereochemistry at this position has little or no effect on anticancer activity.[24] Moreover, edelfosine (Figure 1) the most studied AEL is used as a racemic mixture.[29]

Compound **1** was synthesized by coupling of glycoside donor **17** to the commercially available lipid alcohol **18** to afford a mixture of α- and β-glycolipid **19** and **20** in a 4:1 ratio, respectively (Scheme 1). The glycoside donor **17** was synthesized from glucosamine hydrochloride **11** in seven steps.

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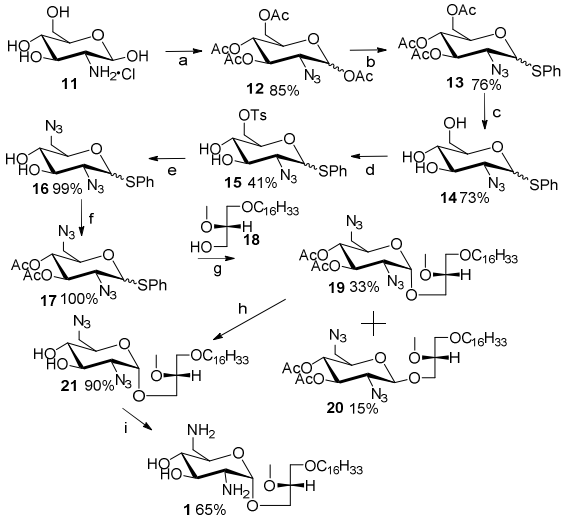
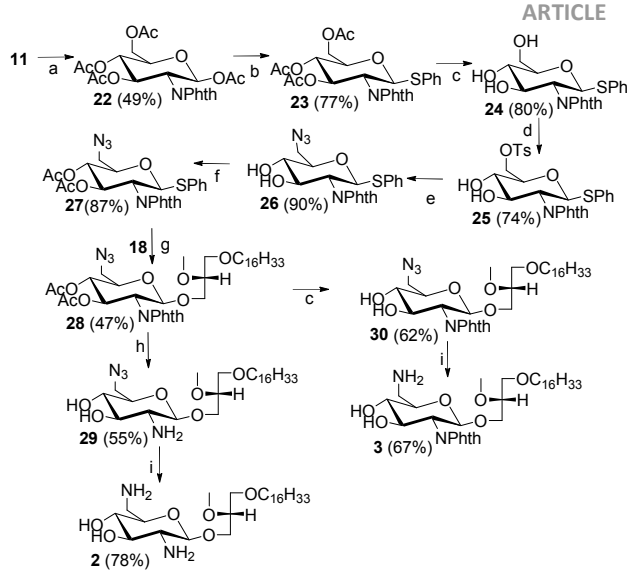


Scheme 1. Synthesis of compound **1.** *Reagents and conditions*: (a) 1. TfN_3, $CuSO_4$, Et_3N, H_2O, rt; 2. Ac_2O, DMAP, pyridine, 18 h, rt (b) PhSH, $BF_3.Et_2O$, DMAP, DCM, 18 h, rt (c) MeONa, MeOH, 1 hr (d) TsCl, pyridine, DMAP, 0 °C to rt, 18 h (e) DMF, NaN_3, 70 °C, 24 h (f) Ac_2O, DMAP, pyridine, 18 h, rt (g) AgOTf, NIS, DCM, 3 h, rt (h) MeONa, MeOH, 30 mins (i) $P(CH_3)_3$, THF, H_2O, 2 h, rt. Abbreviations: 4-dimethylaminopyridine (DMAP), dichloromethane (DCM), 2,2,*N,N*-dimethylformamide (DMF), room temperature (rt) 23 °C, *N*-iodosuccinimide (NIS), triisopropyl benzyl sulphonate ester (OTIBs).



Scheme 2. Synthesis of compounds **2** and **3**. *Reagents and conditions*: (a) 1. Phthalic anhydride, NaOH, H_2O, 18 h, rt; 2. Ac_2O, DMAP, pyridine, 18 h, rt (b) PhSH, $BF_3.Et_2O$, DMAP, DCM, 18 h, rt (c) MeONa, MeOH, 20 mins (d) TsCl, pyridine, DMAP, 0 °C to rt , 18 h (e) DMF, NaN_3, 70 °C, 24 h (f) Ac_2O, DMAP, pyridine, 18 h, rt (g) AgOTf, NIS, DCM, 3 h, rt (h) ethylenediamine/butanol (1:1), 90 °C, 2 h, rt (i) $P(CH_3)_3$, THF, H_2O, 2 h, rt. Abbreviations: 4-dimethylaminopyridine (DMAP), dichloromethane (DCM), 2,2,*N,N*-dimethylformamide (DMF), room temperature (rt) 23 °C, *N*-iodosuccinimide (NIS).

At first, glucosamine **11** was converted to azide **14** as previously reported.[23] The azido function at the C-6-position was installed by selective activation of the C-6 hydroxyl group in **14** as sulphonate ester **15,** followed by nucleophilic displacement of the sulphonate group by sodium azide in DMF to afford the 2,6-diazido analog **16**. Protection of the remaining hydroxyl groups using acetic anhydride in pyridine produced the glycoside donor **17**. Donor **17** was used in glycosylation reaction with commercially available lipid alcohol **18** to produce a mixture of the glycolipids **19** and **20** in ratio 7:3. The α-glycolipid **19** was isolated in pure form and the ester groups were deprotected to afford the 2,6-diazido compound **21** which was subsequently subjected to azide reduction using trimethylphosphine in THF to produce the desired α-anomeric glycolipid **1** (Scheme 1).

Glycolipids **2** and **3** were prepared by coupling of lipid alcohol **18** to thioglycoside donor **27** (Scheme 2). Donor **27** was prepared from glucosamine hydrochloride **11** in seven steps. **11** was converted into the unprotected thioglycoside **24** using established procedures.[23] The azido group at C-6 was installed by selective activation of the 6-hydroxy group as sulphonate ester **25** followed by nucleophilic displacement with sodium azide in DMF to produce azido analog **26** which was acylated to afford the desired thioglycoside donor **27**. Donor **27** was coupled to lipid alcohol **18** to produce glycolipid **28**. Removal of the acetate- and phthalimido-based protecting groups was achieved by exposure to ethylenediamine in butanol (1:1 v/v) at high temperature to yield azide **29** which was subsequently reduced to the desired dicationic glycolipid **2**. To synthesize the target compound **3**, the acetate protecting groups of compound **28** were selectively removed using catalytic amount of sodium methoxide in methanol to give 6-azido-2-phthalimido analog **30**. Reduction of the azido group via Staudinger reaction produced glycolipid **3** (Scheme 2).

To synthesize compound **4**, the glycoside donor **27** was glycosylated to lipid alcohol 31,[23] to produce glycolipid **32** (Scheme 3). Deblocking of the acetate and phthalimido protecting groups produced azide **33** which was reduced to the desired diamine-based glycolipid **4**. Target molecule **5** was synthesized by glycosylating commercially available lipophilic diol **34** to thioglycoside donor **27** to afford protected diglycosylated lipid **35**. Deblocking of diglycosylated lipid produced bisazido compound **36** which was subsequently reduced to produce tetraamine-based glycolipid **5** (Scheme 3). Compounds **6** and **7** were synthesized as outlined in Scheme 4. Azido lipid alcohol **39** was synthesized from commercially available lipophilic diol **37** in two steps. First, the primary hydroxyl group in diol **37** was selectively activated as sulphonate ester **38** followed by a S_N2-type displacement with sodium azide to produce azido acceptor **39**. The glycoside donor **23** was glycosylated with acceptor **39** to afford the protected glycolipid **40**.

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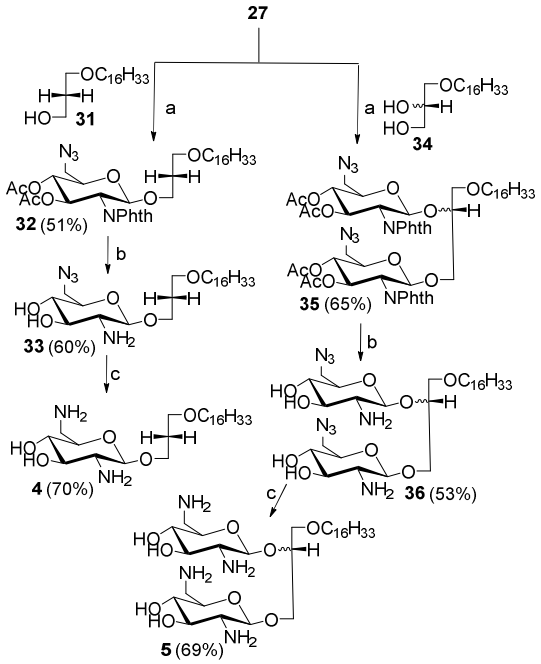
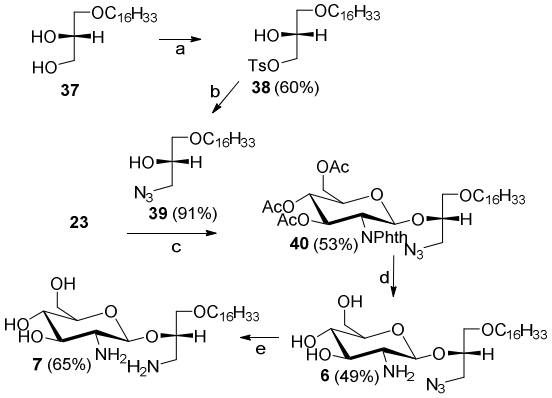
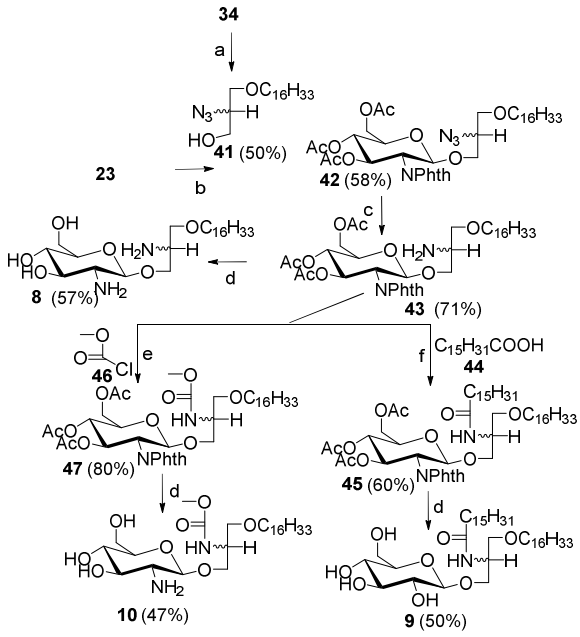


Scheme 3. Synthesis of compounds **4** and **5**. *Reagents and conditions:* (a) AgOTf, NIS, DCM, 3 h, rt (b) ethylenediamine/butanol (1:1), 90 °C, 2 h, rt (c) $P(CH_3)_3$, THF, H_2O, 2 h, rt. Abbreviations: dichloromethane (DCM), 2,2,*N*,*N*-dimethylformamide (DMF), room temperature (rt) 23 °C, *N*-iodosuccinimide (NIS).

The acetate and phthalimido protective groups of **40** were removed with ethylenediamine in butanol (1:1) at 90 °C for 2 h to give the desired compound **6**. The azido substituent of **6** was reduced as described above to afford the diamino analog **7** (Scheme 4).



Scheme 4 Synthesis of compounds **6** and **7**. *Reagents and conditions:* (a) TsCl, Pyridine, DMAP, 0 °C to rt, 18 h (b) DMF, NaN_3, 70 °C 24 h (c) AgOTf, NIS, DCM, 3 h, rt (d) ethylenediamine/butanol (1:1), 90 °C, 2 h, rt (e) $P(CH_3)_3$, THF, H_2O, 2 h, rt. Abbreviations: 4-dimethylaminopyridine (DMAP), dichloromethane (DCM), 2,2,*N*,*N*-dimethylformamide (DMF), room temperature (rt) 23 °C, *N*-iodosuccinimide (NIS).



Scheme 5. Synthesis of compounds **8**-**10**. *Reagents and conditions:* (a) DIAD, Ph_3P, Me_3SiN_3, DCM, 8 h (b) AgOTf, NIS, DCM, 3 h, rt (c) $P(CH_3)_3$, THF, H_2O, 2 h, rt (d) ethylenediamine/butanol (1:1), 90 °C, 2 h, rt. (e) Et_3N, $ClCO_2Me$, DCM, 15 h (f) TBTU, DIPEA, DMF, 3 h, rt. Abbreviations: dichloromethane (DCM), 2,2,*N*,*N*-dimethylformamide (DMF), room temperature (rt) 23 °C, *N*-iodosuccinimide (NIS), diisopropylethylamine (DIPEA), diisopropyl azodicarboxylate (DIAD).

Bisamino glycolipid **8**, was synthesized according to Scheme 5. The lipid alcohol **41**, was synthesized from commercially available lipid alcohol as previously reported,[16] and coupled to the glycoside donor **23** to afford protected glycolipid **42** (Scheme 5). Reduction of the azido substituent in **42** gave compound **43** which was subsequently deprotected using ethylenediamine in butanol (1:1) at elevated temperature to yield dicationic glycolipid **8**. To synthesize glycolipid **9**, the amine **43** was coupled to palmitic acid **44** by condensation with TBTU,[30] to give compound **45** which was deprotected to produce target glycolipid **9** (Scheme 5). Finally, compound **10** was synthesized by reaction of amine **43** with methyl chloroformate **46** to produce carbamate **47** which was subsequently deblocked to afford the desired carbamate-based glycolipid **10** (Scheme 5).[18]

Biological Studies

In vitro screening of GAELs' activity against epithelial cancer cell lines

The cytotoxicity of compounds **1** – **10** was evaluated against exponentially growing epithelial cancer cell lines including BT-474, JIMT-1 and MDA-MB-231 (breast), DU-145 and PC3 (prostate), and MiaPaCa2 (pancreas). Compounds

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were incubated with varying concentrations of **1 – 10** (0 – 30 μM) for 48 h, followed by determination of cell viability using MTS assay.[22] The results of the viability studies are shown in Figure 2. Lead compound **β-GLN,** the most studied GAEL to date, was selected as the reference compound for comparison. The CC_{50} values for all compounds and CC_{90} values for the most active analogs are summarized in Tables 1 and 2. The most potent GAEL amongst the compounds tested against six cell lines is dicationic glycolipid **1,** with CC_{50} values of 3.0 to 7.5 μM, while 90 % loss of cell viability was observed at a concentration range of 4.5 to 9.5 μM, depending on cell lines. This compound bears two primary amino groups at positions 2 and 6 of the glucose moiety and has a α-glucosidic linkage to the glycerolipid. The next most potent analog was compound **2** which differs from **1** merely in its β-glucosidic linkage, with a typical 1.5- to 3-fold less activity relative to **1** (Table 1). This is consistent with our previous results in the monocationic GAEL series, which showed that **α-GLN** was more active than its β-anomer.[22,23] Comparison of the β-dicationic analog **2** with β-monocationic analog **β-GLN** indicates that the dicationic GAEL showed better cytotoxicity than its monocationic analog except against BT-474 cell lines. Compound **3** containing an uncharged phthalimido group at the C-2 position showed weak antitumor activity against all six cell lines ($CC_{50} \geq 15$ μM), indicating that the primary amino group at the C-2 position is crucial for antitumor activity. GAEL **4**, an analog of **2** without a methoxy substituent at the glycero moiety, displayed comparable activity to **2**. Statistical analysis indicated that there was no significant difference between the activity of **2** and **4** across all cell lines except for BT-474 cells. This suggests that the methoxy substituent is not essential for manifestation of antitumor activity in the β-anomeric dicationic glycolipids. This is consistent with previous SAR studies on monocationic, 2-amino-D-gluco-based GAEL that demonstrated the methoxy group as not being crucial for antitumor activity.[22] However, tetracationic glycolipid **5** which was generated by replacement of the methoxy substituent at the *sn*-2 position by a second 2,6-diamino-β-D-gluco residue had a greatly reduced activity and was unable to achieve 50 % loss of cell viability at the highest concentration (30 μM) tested. This may be due to its increased hydrophilicity leading to reduced cellular absorption.

Compounds **6 – 8** were synthesized primarily to determine the effect of the position of the sugar on the glycerolipid. The azido compound **6** has its CC_{50} values in the range of 6 - 22 μM. It was more cytotoxic against PC-3 cell lines than **β-GLN;** CC_{50} of 6.0 μM compared to 13.5 μM, but less against BT-474, (22 μM and 8 μM respectively). For other cell lines, their activities are comparable. The dicationic GAEL **7** with a primary amino group at the *sn*-3 position of the glycerolipid is less potent than mono-cationic **6** across all cell lines. This indicates that the positioning of the second amino group on the glycerol moiety is not optimal. Compound **8** with the sugar moiety on *sn*-3 position of the glycerolipid, was significantly more active compared to compound **7** with the aminosugar on *sn*-2 position of glycerol. GAEL **8** was also more active than **β-GLN** against all the cell lines with the exception of the BT-474 cell line. This result indicates that activity of this class of compounds is better when the sugar moiety is on *sn*-3 position of the glycerolipid.

The increased potency of compound **8** compared to **β-GLN** against most of the cell lines also shows that replacement of the methoxy group at *sn*-2 position of the glycerolipid with amino substituent enhanced cytotoxicity.

Comparison of the activity of compounds **7** and **8** with that of **1**, **2**, and **4** demonstrates that better activities can be achieved when both amino groups are placed on the sugar as compared to a combination where one amino group is present at the sugar and the other one is attached to the glycerol portion of the lipid.

Compound **9** with a second hydrophobic C-16 moiety attached to the *sn*-2 position of the glycerolipid via an amide linkage was not active at the highest concentration tested, 30 μM. At this concentration, there was no statistically significant difference in viability of cells incubated with **9** compared to vehicle-treated cells (controls). The lack of activity might be caused by increased lipophilicity which exceeds the lipophilicity-hydrophilicity balance required for optimal cellular absorption of this compound.

Compound **10** with a methyl-carbamate substituent at the *sn*-2 position of the glycerolipid, was synthesized to potentially promote selectivity for prostate cancer cell lines. This is consequent upon previous report that describes edelfosine analog bearing carbamate at *sn*-2 position of glycerolipid as being more selective towards prostate cancer cell lines.[28] The CC_{50} values of compound **10** which is in the range of 14 – 23 μM is much less active than that of **β-GLN** indicating that the carbamate substituent neither increases activity nor promoted selectivity for prostrate cell line in GAEL series.

It is noteworthy that the most active analogs **1, 2, 4** and **8** were 2- to 3-fold more toxic toward the drug-resistant cancer cell lines, JIMT-1, MB-MDA-231, DU-145, and PC-3, relative to **β-GLN** (Table 1). The reason(s) for the indistinguishable activities of **1** and **β-GLN** against BT-474 cells is unclear.

To compare the cytotoxic activity of our synthesized compounds with that of drugs in clinical use or development, we tested chlorambucil, cisplatin and salinomycin against all the six cell lines used in this study (Table 1). Chlorambucil, a DNA alkylating agent, did not kill up to 50 % of all cell lines at the highest concentration (150 μM) tested. Similarly, we were unable to reach CC_{50} values for cisplatin against BT-474, MDA-MB-231 and MiaPaCa2 cell lines at the highest dose (20 μM) tested. In the case of salinomycin, an experimental drug with CSCs killing properties, we were able to demonstrate that the most potent GAEL **1** displayed > 6-fold activity against MDA-MB-231, > 4-fold activity against DU-145, 2-fold higher against MiaPaCa2 and BT-474. Improved activity over salinomycin was also observed with GAELs **2**, **4** and **8**.

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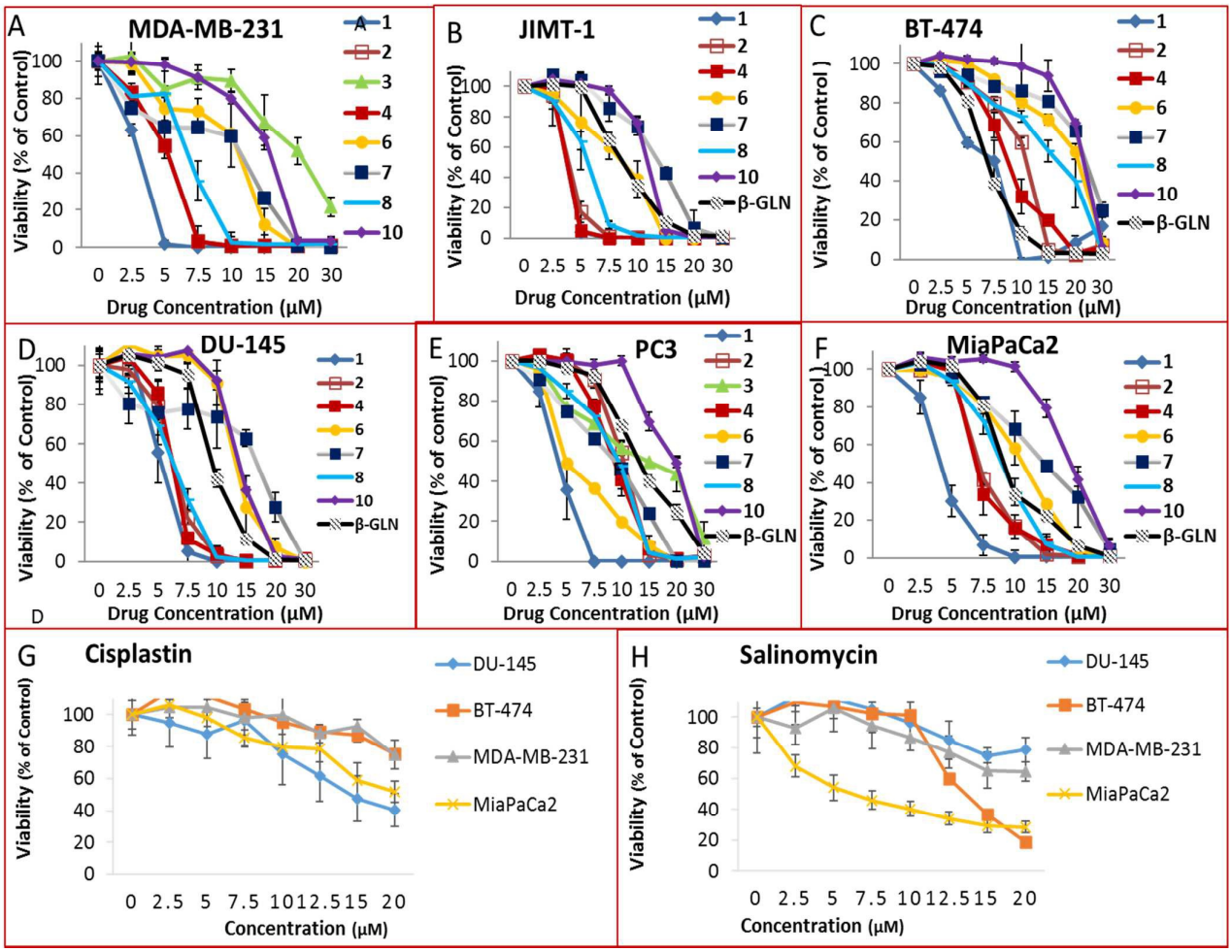

Figure 3. Effects of compounds **1** - **10**, and **β-GLN**, on the viability of MDA-MB-231 (A), JIMT-1 (B), BT-474 (C), DU-145 (D), PC-3 (E) and MiaPaCa2 (F) cell lines. Effect of cisplatin (G) and salinomycin (H) on viability of DU-145, BT-474, MDA-MB-231 and MiaPaCa2 were included for comparison. MDA-MB-231, JIMT-1, DU-145 and MiaPaCa2 cells were cultured in DMEM medium supplemented with 10 % FBS, BT-474 cells were cultured in DMEM/F12 medium supplemented with 10 % FBS and PC-3 cells were cultured in F12K medium supplemented with 10 % FBS. Equal numbers were dispersed into 96-well plates. After 24 h, the cells were incubated with compounds **1** – **11** (0 – 30 μM) for 48 h. At the end of the incubation, MTS reagent (20 % v/v) was added and the plates were incubated for 1 – 4 h. The OD_{490} was read with a plate reader. Wells with media but no cells were treated in similar fashion and the values utilized as blank. The results represent the mean ± standard deviation of 6 independent determinations. Compounds **5, 8, 9**, and **10** were studied as diastereomeric 1:1 mixture based on the stereochemistry at *sn*-2 position of the glycerolipid.

Effect of dicationic GAELs 1, 2, 4, or 8 on the integrity and viability of BT-474 and DU-145 cancer stem cells (CSCs)

To assess the effect of the most potent GAELs on cancer stem cells (CSCs), high expressing aldehyde dehydrogenase 1 (ALDH1) cells were sorted from BT-474 and DU-145 cells, and cultured in low-adhesion culture plates in stem cell growth media. The spheroids were isolated by cell sieving, trypsinised and counted. Equal numbers were dispersed in wells and allowed to form spheroids. The active dicationic GAELs, **1**, **2**, **4**, or **8** were added to the spheroids.

Figures 4A and 5A show the results of the effects of GAELs on the integrity and viability of BT-474 CSC spheroids after 6 days of treatment. Incubations with the vehicle revealed the spheroids grew larger and more compact. In contrast, incubations with the GAELs resulted in disintegration of the spheroids. Similar results were obtained with CSCs derived from DU-145 prostate cancer cell lines (Figures 4B and 5B). The GAELs were able to completely inhibit the viability of the CSCs and disintegrate the CSCs spheroids at concentrations ranging from 5 – 10 μM depending on the compound.

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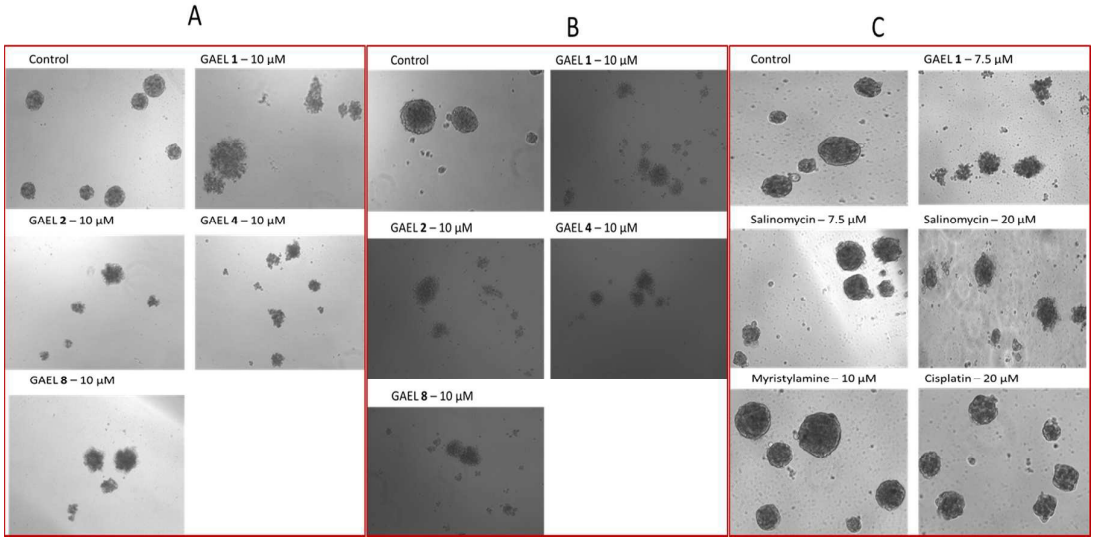

Figure 4. Effect of GAEL compounds **1**, **2**, **4**, and **8** on the integrity of (A) BT-474 breast cancer stem cell spheroids, (B) DU-145 prostate cancer stem cells spheroids, and (C) comparison of effects of **1**, salinomycin, myristylamine and cisplatin on BT-474 CSCs. Equal numbers of BT-474 or DU-145 cancer stem cells were seeded into ultra-low adhesion 48-well plates and grown for 5 days to allow for spheroid formation. The spheroids were incubated with or without 10 µM GAELs for up to 6 days (A and B) or 3 days (C). The images were taken after 4 days (A and B) or 3 days (C) of incubation with an Olympus I×70 microscope (magnification ×10).

The most active compounds were **1**, **2** and **4** for BT474 CSCs and **1**, **4** and **α-GLN** for DU-145 CSCs. When compared to salinomycin, an investigational anti-CSCs drug; cisplatin, a clinical anticancer agent, and myristylamine, a known surfactant, the activity of the most active analog **1** against BT-474 CSCs was superior (Figure 4 and 5).

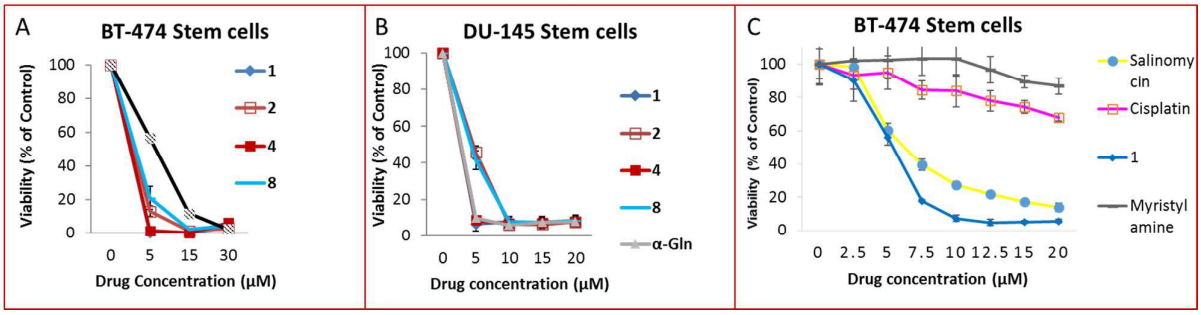

Figure 5. Effects of compounds **1**, **2**, **4**, **8**, **α-GLN** on the viability of cancer stems cells isolated from BT-474 breast cancer (**A**), DU-145 cell lines (**B**) and comparison of **1** with salinomycin, cisplatin, myristylamine (**C**). **α-GLN** has been previously reported to be more active than our reference **β-GLN** against cancer cell lines[22, 23] and CSCs obtained from BT-474 cell line.[22] Also it is being tested on CSCs from DU-145 for the first time. BT-474 and DU-145 cancer stem cells were obtained by staining for ALDH1 and sorting the cells by flow cytometry. The spheroids from BT-474 stems cells were grown in ultra-low adhesion plates in mammocult medium for 6 days, while the spheroids from DU-145 stem cells were grown in ultra-low adhesion plates in prostatosphere growth medium for 6 days. The spheroids formed were harvested, trypsinised and equal numbers were seeded in 48-well low adhesion plates for 5 – 6 days to allow formation of spheroids. The spheroids were incubated with varying concentrations of compounds **1, 2, 4, 8** (0 - 30 µM) for 6 days (**A** and **B**) and 3 days (**C**). At the end of the incubation, the MTS reagent was added to each well and the plates were incubated in a 5 % CO_2 incubator for 4 h. The absorbance was read at 470 nm in a plate reader. The results are the means ± standard deviation for 4 independent determinations (**A** and **B**) and 6 independent determination (**C**).

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Table 1. CC_{50} (A) and CC_{90} (B) values of compounds 1-10, cisplatin, salinomycin, chlorambucil and **β-GLN** on a panel of human epithelial cancer cell lines: breast (BT474, JIMT1, MDA-MB-231), pancreas (MiaPaCa2) and prostrate (DU145, PC3). The CC_{50} value is defined as the concentration required to decrease cell viability by 50% relative to the untreated control, while the CC_{90} value is defined as the concentration required to decrease cell viability by 90% relative to untreated control. The values were obtained by estimating the drug concentration at 50% and 10% viability on the y-axis using line plots. Compounds **5, 8,9,** and **10** were studied as diastereomeric mixtures (1:1) based on the stereochemistry at *sn*-2 position of the glycerolipid. NT – Not tested, ND – Not determined.

	A		CC_{50} values (μM)				B		CC_{90} values (μM)			
Compd	**MDA-MB-231**	**DU-145**	**JIMT-1**	**MiaPaCa2**	**PC-3**	**BT-474**	**MDA-MB-231**	**DU-145**	**JIMT-1**	**MiaPaCa2**	**PC-3**	**BT-474**
1	3.0 ± 0.1	5.2 ± 0.3	3.5 ± 0.2	3.5 ± 0.1	3.5 ± 0.1	7.5 ± 0.3	4.5 ± 0.1	7.4 ± 0.1	4.9 ± 0.1	6.5 ± 0.1	6.0 ± 0.2	9.5 ± 0.1
2	5.5 ± 0.2	6.0 ± 0.2	4.2 ± 0.1	7.0 ± 0.3	11.0 ± 0.3	11.5 ± 0.5	7.0 ± 0.1	8.5 ± 0.1	6.5 ± 0.2	12.0 ± 0.3	14.0 ± 0.1	14.0 ± 0.1
3	20.0 ± 2.1	>30			15.0 ± 0.5	NT	ND					
4	4.5 ± 0.1	6.0 ± 0.1	4.0 ± 0.3	6.5 ± 0.5	8.0 ± 0.9	8.5 ± 0.2	7.0 ± 0.1	8.5 ± 0.1	6.0 ± 0.1	13.0 ± 0.1	14.0 ± 0.2	17.5 ± 0.1
5	>30						ND					
6	11.0 ± 0.1	12.5± 0.6	9.5 ± 0.2	11.5 ± 0.1	6.0 ± 0.1	22.0 ±1.1	15.0 ± 0.3	18.0± 0.5	13.5 ± 0.1	18.5 ± 0.3	15.0 ± 0.1	28.0 ± 0.5
7	12.0 ± 0.4	17.5± 0.4	14.0 ± 0.1	15.0 ± 0.7	9.5 ± 0.3	25.0 ± 0.4	17.5 ± 0.2	25.0± 1.0	19.0 ± 0.5	28.0 ± 1.3	17.0 ± 0.4	>30
8	6.0 ± 0.2	6.0 ± 0.1	5.5 ± 0.1	8.5 ± 0.3	9.0 ± 0.1	15.5 ± 0.4	9.0 ± 0.1	9.0 ± 0.1	7.5 ± 0.1	14.0 ± 0.1	14.0 ± 0.1	28.0 ± 0.8
9	>30						ND					
10	16.0 ± 0.3	14.0± 0.1	12.5 ± 0.1	18.0 ± 0.5	20.0 ± 0.3	23.0 ± 0.8	19.0 ± 0.4	19.0 ± 0.5	15.0 ± 0.2	29.0 ± 0.9	29.0 ±1.0	29.0 ± 1.5
β-GLN	NT	10.0± 0.5	9.0 ± 0.1	9.0 ± 0.1	13.5 ± 0.2	8.0 ± 0.3	NT	15.0± 0.4	16.0 ± 0.2	18.0 ± 0.6	28.0 ± 0.9	13.0 ± 0.5
Cisplatin	>20	14.8± 0.3	NT	>20	NT	>20	ND					
Salinomycin	>20	>20	NT	6.5 ± 0.1	NT	14.0 ± 0.3						
Chlorambucil	>150											

Compound **1** demonstrated a better anti-CSC activity than salinomycin. Cisplatin did not show any significant reduction in viability at highest dose tested (20 μM). Myristylamine, a classical amphiphile did not show any activity at the highest dose tested (20 μM) indicating that the disruption of mammosphere formed by BT-474 CSCs is not due to detergent effect.

Discussion and Conclusion

GAELs represent a novel class of potential anticancer agents. The most active compounds reported herein display CC_{50} values in the range of 3.0 to 7.5 μM. Since GAELs with a primary amino substituent at the C-2 position of the sugar moiety shows higher toxicity against cancer cells compared to analogs without amino function,[18] we explored the possibility that increasing the number of cationic charges would lead to

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enhanced cytotoxic activity. Our results demonstrated that this was indeed the case. Diamino GAELs **1**, **2**, **4** and **8** but not bisamino GAEL **7** were 2- to 3-fold more active than our previous monobasic lead structure **β-GLN**. The fact that GAEL **7** which has the carbohydrate moiety linked at the *sn*-2 position shows reduced antitumor activity when compared to other dibasic GAELs may indicate that the carbohydrate moiety should be linked to the *sn*-3 position of the glycerolipid for optimal antitumor activity. Within the class of diamine-based GAELs, glycolipid **1,** containing an α-glucosidic-2,6-diamino head group, consistently showed the highest activity against all 6 cancer cell lines, indicating that the α-glucosidic linkage and positioning of the two cationic charges in the glucose moiety are optimal for anticancer activity. The lack of activity of compound **3** in comparison to **β-GLN** despite the free amino group at the C-6 position of glucose indicates that the amino group at C-2 remains critical for anticancer activity. Comparison of the activity of GAELs **6** and **7** with that of compound **8** showed that glycosylation of the hydroxyl group at the *sn*-3 position of the glycero moiety appears to be optimal for antitumor activity. The significantly higher activity of compound **2** when compared to that of compound **8** indicates that the presence of both amino substituents on the glucose moiety is optimal for anticancer activity. Similar to previous findings in the monocationic GAEL series,[22,23] replacing the methoxy substituent in the glycero portion by a H-atom had little effect on the cytotoxicity against most cancer cell lines except the PC-3 and BT-474 cell lines where a slight improvement was observed. On the other hand, replacement of the methoxy substituent by a second β-glycosidic 2,6-amino-D-glucosyl moiety as in **5**, or acylation of GAEL **8** by palmitic acid to afford **9**, resulted in a loss of antitumor activity. While the exact reasons for the loss of activity are not clear, the substitutions could affect the physical properties of the compound which may impact absorption and thus the differential in activity. Unlike edelfosine, replacement of the methoxy group at *sn*-2 position of the glycerolipid with methyl carbamate as in **10** significantly reduced anticancer activity and it did not enhance selectivity toward prostate cancer.

Juxtaposing the cytotoxicity of **1**, cisplastin and salinomycin, the most researched ant-CSCs agent,[31] against BT-474, DU-145 and MDA-MB-231 cancer cell lines and CSCs isolated from BT474 cell lines, it is clear that GAELs exhibited the best anticancer properties (Figure 3 G, 3H, 4C and 5C). Interestingly, the classical amphiphile myristylamine did not decrease the viability and disintegrate CSC spheroids, indicating that the anti-CSCs properties of GAELs is not the result of a detergent effect.

Despite the large number of chemotherapeutic agents in clinical use for the management of cancer, the overall treatment outcomes have at best been disappointing. There are compelling evidences that tie tumor relapse to the inability to eradicate CSCs.[6,7,22] A major goal of current cancer research is to identify mechanisms and/or means of killing or inactivating CSCs.[7] Several approaches to curtail the activity of CSCs in tumors have been suggested[7,32-35] but because of their capacity to resist apoptotic cell death, it has been challenging to eradicate them using conventional chemotherapeutic agents. Thus, compounds that kill cells by non-apoptotic mechanisms are attractive potential anti-CSC agents, as they could by-pass a variety of strategies adapted by CSCs to evade apoptotic cell death.

In summary, the current study has demonstrated the ability of glucose-configured diamine-based GAELs to disintegrate and kill CSC spheroids. The retention of this ability across board demonstrates that the various structural modifications made to generate the diamine-based glycolipids did not fundamentally alter their mechanism of action. Thus, the ability to disrupt the integrity of CSCs may be a general characteristic of GAELs.

Experimental

Materials and methods: Synthesis of GAELs

Solvents were dried over CaH_2. ^1H, ^{13}C NMR spectra were recorded with a JMN A500 FT NMR spectrometer at 500 or 300 MHz and at 125 or 75 MHz, respectively, and chemical shifts were reported in parts per million (ppm) relative to the indicated solvent Thin-layer chromatography (TLC) was carried out on an aluminum-backed silica gel GF plates (250 mm thickness) and plates were visualized by charring with 5 % H_2SO_4 in MeOH and/or short wavelength UV light. Compounds were purified by flash chromatography on silica gel 60 (230-400 ASTM mesh). Matrix assisted laser desorption ionization-time of flight (MALDI-TOF)-MS was recorded on a voyager RP mass spectrometer. High-resolution (HR) mass spectra were recorded on a JEOL JMS700 under FAB conditions. Purity of compounds **1 – 10** were assessed by elemental analysis, and were within ± 0.5 % of the calculated theoretical values.

Chemistry:

General method for acetylation

Acetylation reaction were carried out in pyridine with catalytic amount of dimethyl amino pyridine (DMAP, 0.2 equiv.) using acetic anhydride (2 equiv.). After stirring for 18 h at room temperature 23 °C, the reaction was stopped by addition of methanol, and concentrated to dryness. The resulting residue was dissolved in ethyl acetate, washed with saturated sodium bicarbonate (×3) and distilled water (×2). The resulting organic layer was dried over Na_2SO_4 and concentrated to dryness. The residue was purified by flash chromatography, with yields ranging from 40 – 80 %.

General method for glycosylation reaction

The glycoside donor and 1.1 molar equivalent of the lipid alcohol, the glycoside acceptor, were dissolved in anhydrous dichloromethane (DCM) under argon atmosphere. N-Iodosuccinimide (NIS, 1.5 equiv) and silver triflate (AgOTf, 0.2 equiv) were simultaneously added. The reaction mixture was stirred for 3 h. Upon completion, the reaction mixture was diluted with DCM and filtered over celite. The resulting organic layer was washed with saturated sodium thiosulphate solution (×2), saturated sodium bicarbonate (×3), and water (×2). The

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organic layer was then dried over anhydrous Na_2SO_4 and concentrated under vacuum to give a residue which was purified by flash chromatography (hexanes/ethyl acetate, 2:3)

General method for conversion of primary hydroxyl group to azide

Triisopropylbenzylsulphonylchloride (TIBS) (1.5 equiv) or *p*-toluenesulphonylchloride (1.1 equiv) were used to activate the primary hydroxyl group at the C-6 position of the sugar or primary hydroxyl group of the glycerolipid-based diol, using DMAP (0.2 equi) as catalyst in anhydrous pyridine under argon or nitrogen atmosphere. The reaction was stirred at rt for 12 to 24 h, after which it was stopped by addition of methanol. The methanol and pyridine were removed under high vacuum. The crude mixture was dissolved in EtOAc and washed with 5 % HCl (×2), saturated sodium bicarbonate solution (×2), and water (×1), to give a dark brown organic layer. The organic solvent was concentrated and purified by flash chromatography (hexanes/ethyl acetate, 2:3) or 100 % ethyl acetate. The sulphonate ester was displaced with azide in a nucleophilic substitution reaction using sodium azide (10 equiv) in anhydrous DMF at 70 – 90 °C under argon or nitrogen atmosphere for 12 to 24 h. Upon completion, the mixture was concentrated, resuspended in ethyl acetate, filtered, re-concentrated, and purified using flash chromatography.

General method for deprotection of acetate group

The acetate protected compounds were dispersed in methanol followed by the addition of a catalytic quantity of NaOMe (0.5 equiv). The mixture was stirred for 1 h and stopped with ion exchange resin (H^+). When the reaction mixture was clear, the resin was filtered, concentrated under vacuum, and purified by flash chromatography (100% ethyl acetate).

For selective deprotection of acetate in the presence of phthalimido protective group, the reaction was stirred for lesser time (20 to 25 mins).

General method for simultaneous deprotection of acetate and phthalimido group

To simultaneously remove acetate and phthalimido protecting group, the protected compound is dissolved in a mixture of ethylenediamine/n-butanol (1:1, v/v) and stirred at 90 °C for 2 h. Then the reaction mixture was concentrated under high vacuum and purified using reverse phase C-18 silica gel column by gradient elution of water and methanol.

General method for reduction of azide

To reduce the azido protecting group to free amine, the azido compounds were suspended in a mixture of THF/water (9:1), then trimethyl phosphine in THF (1 M, 5 – 10 equiv) was added. The reaction was stirred at rt for 2 h and concentrated under vacuum. Residues of final compounds were purified using C-18 coated silica gel column chromatography by gradient elution of water and methanol, while the lipid molecule with azido group was purified using normal phase flash chromatography (hexanes/ethylacetate (9:1, v/v) and 100% ethyl acetate for protected glycolipid).

Synthetic procedures for the synthesis of intermediates and final compounds

See supplementary information

Biological methods

Effect of GAELs on viability of epithelial cancer cell lines

The cell lines were cultured from frozen stocks originally obtained from ATCC. MDA-MB-231, JIMT-1 and DU-145 were grown in DMEM medium supplemented with 10 % FBS. BT-474 cells were grown in DMEM/F12 medium supplemented with 10 % FBS. MiaPaCa-2 was cultured in DMEM supplemented with 10 % FBS and 2.5 % horse serum. PC-3 cells were cultured in F12K medium supplemented with 10 % FBS. All the media contained penicillin/streptomycin.

The effects of the GAELs on the viability of the various epithelial cancer cell lines was determined as previously described.[12,13,16] Briefly, equal numbers of the cells were dispersed into 96-well plates. After 24 h, the cells were incubated with the compounds (0 – 30 μM) for 48 h. At the end of the incubation, MTS reagent (20 % v/v) was added and the plates were incubated for 1 – 4 h in a CO_2 incubator. The OD_{490} was read with a plate reader (Molecular Devices). Wells with media but no cells were treated in similar fashion and the values utilized as blank. The results represent the mean ± standard deviation of 6 independent determinations.

Isolation of breast cancer stem cells from BT-474 and prostate cancer stem cells from DU145 cell lines and determination of the effect of GAELs on the viability of the cancer stem cells.

A population enriched in BT474 breast cancer stem cells or DU-145 prostate cancer stem cells was obtained by staining the cells for aldehyde dehydrogenase using the Aldefluor assay kit from Stem Cell Technologies (Vancouver, BC, Canada) according to the manufacturer's instructions, with appropriate controls. The stained cells were sorted from the bulk population by flow cytometry on a 4 laserMoFloXPP high speed/pressure cell sorter. The cells were pelleted by centrifugation. BT-474 cells were resuspended into ultra-low adhesion plates in mammocult medium (Stem cell Technologies), and DU-145 stem cells in their growth medium (DMEM/F12 medium supplemented with 20 ng/ml EGF, and 10 ng/ml basic FGF, 5 μg/ml insulin, 0.4% BSA, with 1% antibiotics).[36] The dishes were incubated at 37 °C in a CO_2 incubator for 4 days for spheroid formation.

The spheres are separated from single cells with a 40 μm nylon cell strainer. The spheres retained in the strainer were washed with PBS and trypsinised to obtain single cells. The cell numbers were counted with a Coulter ZM counter and the cells were dispersed into 48-well low adhesion plates (Grenier) in a volume of 500 μl. The cells were incubated for 4 days to allow for formation of spheroids. Subsequently, the stock

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GAELs in ethanol were diluted to twice the final concentration in the media and a volume of 500 µl was added to the wells. Wells with growth medium but no cells were treated similarly as wells with cells. After 5 days incubation, MTS reagent (2 % v/v) was added to each well and the plates were incubated for 1 – 4 h for formation of colour. The OD_{490} were read in a Molecular Device absorbance plate reader using the SpectroMax software.

Statistical analysis

The results represent the mean ± standard deviation of 6 independent determinations. Statistical significant difference tests were carried out using GraphPadInstat software. The mean values were subjected to one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests as post hoc test. Comparisons were carried out between the viability of controls and drug treated cells to determine if statistically significant differences existed between the two groups. The results of the effects of different concentrations of the compounds were also compared for statistically significant differences to determine if the cytotoxic activities of the drugs are dose dependent. The anticancer activities of all the compounds **1 – 10** tested and the lead compound **β-GLN** was also compared using ANOVA, followed by Turkey-Kramer multiple comparison tests at the following concentrations: 5, 7.5 and 10 µM to determine statistical significance in potency. A *p*-value > 0.05 indicates no statistical differences while a *p*-value of < 0.001 indicated statistical significant differences. The statistical analysis data are not included in this report.

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