In vitro anticancer activity and mechanism of action of an aziridinyl galactopyranoside

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ABSTRACT We recently screened a series of new aziridines β-D-galactopyranoside derivatives for selective anticancer activity and identified 2-methyl-2,3-[N-(4-methylbenzenesulfonyl)imino]propyl 2,3-di-O-benzyl-4,6-O-(S)-benzylidene-β-D-galactopyranoside (AzGalp) as the most promising compound. In this article, we explore possible mechanisms involved in the cytotoxicity of this aziridine and evaluate its selective anticancer activity using cancer cells and normal cells from a variety of tissues. Our data show that AzGalp induces DNA damage (detected with the comet assay). Cells deficient in the DNA repair pathway nucleotide excision repair (NER) were hypersensitive to the cytotoxicity of this compound. These results suggest that AzGalp induces bulky DNA adducts, and that cancer cells lacking a functional NER pathway may be particularly vulnerable to the anticancer effects of this aziridine. Several experiments revealed that neither the generation of oxidative stress nor the inhibition of glycolysis played a significant role in the cytotoxicity of AzGalp. The combinations of AzGalp with either oxaliplatin or 5-fluorouracil slightly improved the ability of both anticancer drugs to selectively kill cancer cells. AzGalp also displayed selective cytotoxicity against a panel of malignant cells versus normal cells; the highest selectivity was observed for two acute promyelocytic leukemia cell lines. Additional preclinical studies are necessary to evaluate the anticancer potential of AzGalp.

KEYWORDS: aziridine, cancer, cytotoxic, cytotoxicity, selectivity, nucleotide excision repair

1. INTRODUCTION Cancer kills millions of people every year, with metastasis being the main cause of cancer-related deaths (1). When the disease is spread and surgery and radiotherapy are no longer curative, pharmacotherapy becomes the main form of treatment. However, the available anticancer drugs do not usually cure the disease because of their limited selectivity towards cancer cells. The low efficacy of the existing anticancer drugs is reflected in the poor survival rates of patients diagnosed with the most common metastatic cancers. For example, over 50% of people diagnosed with lung cancer have distant metastasis at the time of diagnosis, and only 5% of them survive five years after diagnosis. Metastasis will continue to be an incurable disease for most patients until we develop therapeutics with a high selectivity against cancer cells (1,2).
Aziridines are highly reactive compounds based on a three-membered heterocycle similar to epoxide but with a nitrogen atom instead of an oxygen. The aziridine group is present in the structure of useful reaction intermediates in organic chemistry and also in a variety of secondary metabolites from plants and microorganisms (3,4). Many of these compounds have displayed anticancer activity (5–8). Indeed, some of them have been used in cancer therapy since decades, such as thiotaepa and mitomycin C (Figure 1). Thiotaepa (tris(1-aziridinyl)phosphine sulfide) is an alkylating agent approved for treatment of breast, ovary and bladder cancer. Although the main mechanism of this drug remains unclear, it is thought that the aziridyl groups of thiotaepa induce cross-links with DNA, interfering with DNA replication and cell division (9). Mitomycin C is an aziridine alkaloid isolated from Streptomyces bacteria (3,10). This anticancer drug is useful for the treatment of gastric and pancreatic adenocarcinomas. It is known that its aziridine moiety displays an important role in its cytotoxicity (10). The cytotoxic mechanism of this alkaloid includes the alkylation of DNA, the generation of oxygen radicals and the inhibition of DNA and RNA synthesis. Other aziridine alkaloids isolated from nature have also displayed anticancer activity (3). These data suggest that compounds containing aziridine in their structure could have potential anticancer activity.

Previously, we carried out a preliminary screening of a new series of aziridines from alkenyl-β-D-galactopyranoside derivatives for selective cytotoxic activity against A549 human lung cancer cell line and MRC-5 human lung normal cell line using the MTT assay (4). Several aziridine-containing compounds showed notable selectivity against A549 lung cancer cell line. The most selective compound, 2-methyl-2,3-[N-(4-methylbenzenesulfonyl)imino]propyl 2,3-di-O-benzyl-4,6-O-(S)-benzylidene-β-D-galactopyranoside (AzGalp, Figure 1), was approximately 6 times more cytotoxic against A549 lung cancer cells than against MRC-5 lung normal cells. In this article, we evaluated the possible mechanisms involved in the cytotoxic activity of AzGalp and assessed its cytotoxicity against a panel of normal and malignant cell lines.

2. MATERIALS AND METHODS

**Chemicals.** Catalase, camptothecin (CPT), 4′,6-diamidino-2-phenylindole (DAPI), dichloroacetate (DCA), etoposide, 5-fluorouracil (5-FU), hydrogen peroxide (H₂O₂), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-acetylcysteine (NAC), oxaliplatin and resazurin were purchased from Sigma. Mn(III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) was obtained from Biomol Laboratories. 2-Methyl-2,3-[N-(4-methylbenzenesulfonyl)imino]propyl 2,3-di-O-benzyl-4,6-O-(S)-benzylidene-β-D-galactopyranoside (AzGalp) was synthesized as described previously (4). All other compounds used in this work were obtained from Sigma. Stock solutions of AzGalp, CPT, etoposide, 5-FU, MnTMPyP and oxaliplatin were prepared in DMSO. Catalase, DCA, H₂O₂, MTT, NAC and resazurin were dissolved in culture medium before use.

**Cell lines.** A549 (human non-small cell lung cancer cells) and MRC-5 (human lung fibroblastic cells) were purchased from European Collection of Cell Cultures. BJ-hTERT (hTERT immortalized foreskin fibroblast BJ cells), BJ-SV40T (SV40T-transformed BJ-hTERT cells), and BJ-RASV12 (HRASV12-transformed BJ-SV40T cells) were kindly provided by Dr. Hahn (Dana-Farber Cancer Institute, USA) (11). HeLa (human cervical carcinoma cells), HepG2 (human hepatocellular carcinoma cells), HL-60 (human acute promyelocytic leukemia cells), NB4 (human acute promyelocytic leukemia cells), PC3 (human prostate cancer cells), SW480 (human colon adenocarcinoma cells), U2OS (human osteosarcoma cells) and VH10 (human foreskin fibroblast cells) were generously provided by Dr. Helleday (Karolinska Institute, Sweden) (12). UACC-62 (human melanoma cells) was obtained from National Cancer Institute (NCI). To study the possible DNA damage response induced by AzGalp, the following parental and DNA repair-deficient cell lines were used: AA8 (parental Chinese hamster ovary cells, DNA repair proficient), UV4 (AA8 cells mutated in ERCC1, nucleotide excision repair (NER)-deficient) and UV5 (AA8 cells mutated in ERCC2 (XPD), NER-deficient). AA8, UV4 and UV5 were a gift from Dr Helleday (Karolinska Institute, Stockholm, Sweden) (13,14). A549, AA8, BJ-hTERT, BJ-RASV12, BJ-SV40T, HeLa, HepG2, MRC-5, SW480, U2OS, UACC-62, UV4, UV5 and VH10 were grown in Dulbecco’s modified Eagle’s medium (DMEM) high glucose medium. PC3 was cultured in DMEM-F12. HL60 and NB4 were maintained as a suspension culture in RPMI 1640. All media were supplemented with 10% fetal bovine serum, 100 U/mL.
penicillin and 100 μg/mL streptomycin. All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2. Cell culture reagents were purchased from Thermo Fisher Scientific.

**Cell viability assays.** Exponentially growing cells were plated in 96-well plates and were allowed to attach and grow during 24 h. Then, the cells were treated with increasing concentrations of the tested drugs. At the end of the treatment period (see the legends of figures for more information), cell viability was measured with the MTT assay or the resazurin assay, two widely used techniques.

In the case of MTT assay, this technique detects viable cells using the ability of these cells to reduce the yellow tetrazolium MTT to an insoluble colored formazan product. Subsequently, this product is solubilized and analyzed by spectrophotometry. Dead cells cannot reduce the MTT into the colored formazan product. Briefly, 24 hours after plating, cells were treated with the tested compounds for 48 h; except in the experiments with the DNA repair-proficient and –deficient cell lines, in which cells were only exposed for 24 h to the drugs and were allowed to grow for additional 48 h in drug-free medium to let them repair the possible DNA damage induced by the tested drugs. After the treatment period, the medium was removed, the cells were washed once with PBS, and 125 μL MTT (1 mg/mL in medium) was added to each well. The plates were incubated for 2-4 hours and, then, 80 μL 20% SDS in 0.02 M HCl was added to each well. Finally, plates were incubated overnight at 37 °C and optical densities were measured at 540 nm using an absorbance spectrophotometer microplate reader.

The resazurin assay is a redox-based colorimetric/fluorometric technique based on the ability of viable cells to reduce the blue compound resazurin into the pink, fluorescent and soluble product resorufin. The amount of resorufin produced is generally proportional to the number of living cells. In this assay, 24 hours after seeding, cells were exposed to the tested drugs for 72 h. Then, 100 μL resazurin in medium were added to each well (final concentration of 10 μg/mL) and, one hour later, fluorescence intensity was measured at 530/590 nm (excitation/emission) using a fluorescence microplate reader.

In both assays, cell viability was expressed as percentage in relation to untreated cells. The results were expressed as the means ± standard error of the mean (SEM). All data are from at least three independent experiments.

**Survival assay.** Cell survival following treatment was determined by clonogenic assay. AA8 and UV4 cells were seeded in 6 cm Petri dishes 4 h prior to treatment for 24 h. After that, drugs were removed and cells were allowed to form colonies for 8 days. Then, colonies were stained with 0.4% methylene blue in methanol for 15 min. Surviving colonies made up of more than 50 cells per colony were counted. The results were represented as percentage of survival referred to untreated cells.

**Comet assay.** The single cell gel electrophoresis assay, also known as Comet assay, is a widely used technique for the study of levels of damage in cellular DNA. In this assay, individual cells embedded in agarose are lysed, electrophoresed, stained with a fluorescent dye and examined under epifluorescence microscope. A look through the microscope shows DNA-damage cells have the appearance of a “comet”, with a head (undamaged DNA nucleoid part) and tail (DNA fragments). The quantity of DNA present in the tail is indicative of DNA damage. This assay has been described in detail by Singh et al (15). We followed this protocol with minor modifications described previously (16). Briefly, standard slides were immersed in 1% normal melting agarose at 55 °C, left to allow the agarose to solidify, and kept at 4 °C until use. Cells were plated in 6-well plates, allowed to grow for 24 h and, then, cells were exposed to AzGalp or CTP (positive control) for 4 h. At the end of the treatment, cells were harvested by trypsinization, washed with PBS and resuspended in PBS at a concentration of approximately 1 million cells/mL. 10 μL cell suspension was mixed with 85 μL of low-melting agarose (LMA) at 37°C and the mixture was rapidly added over the slides with the first agarose layer, spread using a coverslip and kept at 4°C for 8 min to allow the LMA to solidify. The coverslips were then removed, and another layer of 100 μL of LMA at 37 °C was added, covered with a coverslip, and allowed to solidify at 4°C for 8 min. After removing the coverslips, slides were incubated in the dark for 1 h at 4°C in a lysis buffer (pH 10.0) containing 0.25 M NaOH, 10% DMSO, 1% Triton X-100, 10 mM Tris-HCl, 100 mM Na2-EDTA and 2.5 M NaCl. Next, alkaline denaturation with electrophoretic buffer (300 mM NaOH and 1 mM Na2-EDTA) was carried out in an electrophoresis chamber for 20 min and, then, electrophoresis was run at 1 V/cm for 20 min. The slides were later neutralized with 3 x 5 min washes of neutralizing buffer (0.4 M Tris-HCl, pH 7.5). Cells were stained with DAPI in Vectashield and images were taken with an epifluorescence microscope. The
analysis of approximately 50 cells/sample was performed with the CometScore software. The levels of DNA damage were quantified for each cell and was expressed as percent of DNA in the tail and as tail moment (defined as the product of the tail length and the fraction of total DNA in the tail). The results were averaged from two independent experiments and were expressed as mean ± SEM.

**Glycolysis inhibition.** Inhibition of glycolysis was determined by measuring glucose consumed (initial product of glycolysis) and lactate produced (final product of glycolysis) in untreated and treated cells. Cells were seeded into 6-well plates at a density of 10⁶ cells/well and were allowed to attach for few hours before treatment with the tested compounds for 8 h. After treatment, medium was recollected and glucose and lactate concentrations were determined by using the Accutrend® Plus analyzer together with Accutrend glucose strips and BM-Lactate strips (Roche Diagnostics). After calibrating the instrument with glucose and lactate calibration strips, test strips were used to determine glucose and lactate levels via colorimetric-oxidase mediator reactions according to the manufacturer's instructions (17). Results are expressed as percentage of glucose consumption and percentage of lactate production in relation to untreated cells. Data were averaged from two independent experiments and were shown as the mean ± SEM.

**Statistical analysis.** For statistical analysis, the t-test (paired, two-tailed) was used. A p value > 0.05 is not considered statistically significant and is not represented by any symbol. A p value < 0.05 is considered to correspond with statistical significance and is indicated with an asterisk (*), a p value < 0.01 is indicated with a double asterisk (**), and a p value < 0.001 is indicated with a triple asterisk (**). When the cytotoxic activity of a drug was determined against two cell lines, the statistical analysis was carried out to compare the cytotoxicity of a particular concentration of the compound between both cell lines.

3. RESULTS

3.1. AzGalp induces DNA damage

Because aziridine-containing compounds are known to induce DNA damage (9,10), we used the comet assay to test whether AzGalp, the most interesting aziridine from our previous screening (4), could induce DNA damage. To obtain an appropriate concentration for the DNA damage assay, AA8 cells were exposed to several concentrations of AzGalp for 24 h and, then, cells were allowed to grow in drug-free medium for 48 h. The percentage of cell viability was determined by the MTT assay; the IC50 value (mean ± SEM) was 397.0 ± 142.1 µM. We chose a concentration below to IC50 value for the comet assay. AA8 cells were exposed to 50 µM AzGalp or 10 µM camptothecin (CPT, a known DNA-damaging agent used as positive control) for 4 h, and the comet assay was carried out to evaluate DNA damage. The results, represented in Figure 2, show that approximately 30 % of cells treated with AzGalp developed higher levels of DNA damage than untreated cells. However, the level of DNA damage induced by AzGalp was lower than that of the DNA-damaging agent CPT.

3.2. Role of nucleotide excision repair in the cytotoxicity of AzGalp

Alkylating agents are known to induce bulky DNA adducts that distort the DNA double helix. This type of DNA damage is usually repaired by nucleotide excision repair (NER) (18,19). Our next goal was to use NER-deficient cell lines to study if this type of DNA damage was involved in the cytotoxicity of AzGalp. UV4 and UV5 cells (NER deficient), and AA8 cells (NER-proficient) were exposed to several concentrations of AzGalp for 24 h and, after 48 h in drug-free medium, cell proliferation was studied by the MTT assay. Figure 3A shows that both NER-deficient cell lines were hypersensitive to the cytotoxicity of this aziridine-containing compound. The IC50 values (means ± SEM; µM) were 318.7 ± 77.2; 58.6 ± 9.3 and 38.6 ± 6.3 for AA8, UV4 and UV5 respectively. These data show that NER-deficient cells were approximately 6 times more sensitive than NER-proficient cells. That hypersensitivity of NER-deficient cells was also observed when we did the clonogenic assay (Figure 3B). UV4 and AA8 cells were treated with 10 µM AzGalp or 3 µM etoposide (positive control) for 24 h and, then, cells were allowed to grow in colonies in drug-free medium for one week. UV4 cells were more sensitive to the cytotoxic effect of AzGalp or etoposide than AA8 cells. All these results suggest that AzGalp may induce DNA adducts, which can be repaired by the NER pathway.
3.3. Evaluation of other possible mechanisms involved in the selective cytotoxic activity of AzGalp

Evidence suggests that reactive oxygen species (ROS) have an important role in the anticancer activity of several clinically useful drugs (20,21). To test whether the ROS generation participates in AzGalp-induced cytotoxicity, A549 cells were exposed to this compound in the presence or absence of the antioxidants catalase, N-acetylcisteine (NAC) and the superoxide dismutase mimetic MnTMPyP for 48 h and cell proliferation was determined with the MTT assay (Figure 4A-C). The cytotoxicity of H2O2 (positive control) decreased in the presence of the three antioxidants. Neither MnTMPyP nor catalase prevented the cytotoxicity of AzGalp. Although NAC slightly prevented the cytotoxic activity, it was not statistically significant. These data suggest that ROS formation does not participate in the anticancer activity of AzGalp.

Because cancer cells generally have a high dependence on glycolysis for proliferation and survival, glycolysis inhibition can trigger selective anticancer effects (22,23). To evaluate whether AzGalp could act as a glycolysis inhibitor, A549 were treated with AzGalp for 8 h and concentrations of glucose (initial product of glycolysis) and lactate (final product of glycolysis) were measured. The glycolysis inhibitor DCA was used as a positive control. DCA reduced lactate production and glucose consumption. However, the glycolytic activity of A549 cells was not affected by AzGalp (Figure 4D), therefore indicating that the cytotoxic activity of this aziridine is not mediated by inhibition of the glycolytic pathway.

3.4. Cytotoxicity of AzGalp in combination with the anticancer drugs 5-fluorouracil and oxaliplatin

Chemotherapy regimens are usually based on the combination of anticancer drugs to improve their specificity and efficacy. We next tested the cytotoxic activity of AzGalp in combination with the anticancer drugs 5-FU (antimetabolite) and oxaliplatin (alkylating agent), two commonly drugs used to treat different types of cancer. MRC-5 and A549 cells were treated with 100 μM AzGalp with and without 10 μM 5-FU or 10 μM oxaliplatin for 48 h. All drugs were added simultaneously when tested in combination. Cell proliferation was determined by the MTT assay. Results are represented in the Figure 5. Although the combination of AzGalp with 5-FU or oxaliplatin killed more cancer cells than when these drugs were administered individually, we did not observed a clear synergistic effect. However, the combinations did not increase the cytotoxicity against the normal cells. The combinations of AzGalp with oxaliplatin or 5-fluorouracil slightly improved the ability of both anticancer drugs to selectively kill cancer cells.

3.5. Determination of cytotoxicity of AzGalp against a panel of cancer cells.

We next evaluated the cytotoxic activity of AzGalp in cancer cell lines derived from solid tumors (HeLa, PC3, U2OS, HepG2 and SW480) and from hematological malignancies (NB4 and HL-60). To study the selectivity against cancer cells, MRC-5 and VH10 cells were used as normal cell lines. 5-FU was used as positive control. All cells were treated with AzGalp or 5-FU for 72 h and cell viability was evaluated with the resazurin assay. The results are collected in Figure 6A-B and Table 1. The leukemia cell lines were approximately 15 times more sensitive than normal cells to the cytotoxicity of our aziridine. AzGalp also displayed selective cytotoxicity against liver cancer HepG2 cells and osteosarcoma U2OS cells, being approximately 2.5-fold more cytotoxic against these cell lines than against the normal cell lines. This compound showed similar cytotoxic effect against HeLa cervical carcinoma cells, PC3 prostate cancer cells and SW480 colon adenocarcinoma cells than against MRC-5 and VH10 normal cells.

We also evaluated the selective cytotoxic activity of AzGalp against cancer cells using a model of malignant transformation in vitro (11,12). This model consists of three genetically modified cell lines: BJ-hTERT, BJ-SV40T and BJ-RASV12. All cells are derived from normal foreskin BJ cells transformed genetically using hTERT (human telomerase reverse transcriptase), SV40LT (Simian virus 40 large T antigen) and HRAS. All these cells express hTERT to avoid senescence. BJ-SV40T and BJ-RASV12 cells express SV40LT that inactives two major tumor suppressors, RB and p53. BJ-RASV12 cells also express HRAS, an oncogene associated with numerous carcinogenic events. In this model, BJ-hTERT cells are considered as non-malignant, BJ-SV40T as pre-malignant and BJ-RASV12 as malignant cells. We observed that AzGalp was more cytotoxic against pre-malignant and malignant cells (Figure 6C). BJ-RASV12 malignant
cells were three times more sensitive to AzGalp than BJ-hTERT non-malignant cells. It is worth mentioning that we did not observe this selectivity with the anticancer drug 5-FU (Figure 6D).

4. DISCUSSION

Patients with metastatic cancers need selective anticancer treatments to improve their poor survival rates (1). We previously screened a series of new aziridines ß-D-galactopyranoside derivatives for selective anticancer activity and identified AzGalp as the most promising compound (4). In this article, we study possible mechanisms involved in the cytotoxicity of this aziridine and evaluate its selective anticancer activity in additional cancer cells and normal cells.

It is known that aziridine-containing compounds (e.g. thiotepa and mitomycin C) and aziridinium intermediates formed during the activation of nitrogen mustards (structures in Figure 1) induce DNA alkylation damage (10). The alkylation is due to the electrophilic character of the aziridine ring that reacts with endogenous nucleophiles such as nitrogenous bases in DNA. This reaction generates DNA adducts that can block the DNA replication and transcription machinery, generating DNA lesions and death cell (10). The reactivity of aziridine ring with DNA bases suggested that the cytotoxic effect of AzGalp could be due to the generation of DNA damage. We therefore initiated our investigation by studying the ability of AzGalp to induce DNA damage. To test this hypothesis, we used the Comet assay, a classic DNA damage detection technique. We observed that cells treated with AzGalp for a short time displayed high levels of DNA damage (Figure 2).

Our next aim was to evaluate if the DNA damage induced by AzGalp played a role in its cytotoxicity. Cells have developed several pathways to repair DNA lesions induced by either endogenous processes (e.g. replication) or exogenous sources (e.g. DNA-damaging drugs). It is known that the type of DNA lesion induced by alkylation agents is generated by DNA adducts which distort the DNA double helix. These adducts are usually repaired by NER. Therefore, cells defective in NER are usually more sensitive to alkylation agents, including aziridine-containing drugs (18,19). We therefore evaluated the role of NER in the cytotoxic effect of AzGalp. Data in Figure 3 show that NER-deficient cells were hypersensitive to AzGalp, suggesting that this aziridine may induce DNA double helix distortion which can be repaired by NER. The hypersensitivity of NER-deficient cells to AzGalp may have therapeutic implications. Evidences suggest that tumor cells have defects in DNA repair pathways which make them incapable of correctly repairing some types of DNA lesions (18,24). These defects may explain why cancer cells are more sensitive than healthy cells to specific DNA-damaging drugs. For example, several studies suggest that the efficacy of platinum compounds and alkylating agents against specific tumors, such as testicular cancer and non-small-cell lung cancer, may be associated with the defects in NER of these tumors (18). Unlike healthy cells, these tumor cells would not be able to repair the type of DNA lesions induced by these drugs and would die. These data suggest that cancer cells defective in NER may be more vulnerable to the cytotoxicity of AzGalp.

Although the generation of DNA damage seems to play an important role in the cytotoxic activity of AzGalp, it may not be the only mechanism of action involved. It is known that anticancer drugs usually kill cancer cells through different pathways. For example, mitomycin C alkylates DNA, generates ROS, and inhibits DNA, RNA and protein synthesis. We carried out several assays to determine the possible participation of other mechanisms of action in the selective cytotoxic activity of AzGalp. The generation of ROS is a common cytotoxic mechanism of anticancer drugs (20,23,25–28). ROS are involved in oxidative DNA damage, mitochondrial damage and apoptosis. To test the contribution of oxidative stress in the cytotoxicity of AzGalp, we evaluated its cytotoxic effect in the presence or absence of antioxidants (Figure 4). We did not observe a significant reduction of cytotoxicity in the presence of antioxidants, thereby indicating that ROS do not participate in the anticancer activity of this aziridine compound. Although NAC slightly prevented the cytotoxicity of AzGalp, this effect may not be necessarily mediated by a ROS-scavenging mechanism. NAC acts directly as oxidant scavenger or indirectly as precursor of glutathione (29). NAC could also protect against toxic agents by direct adduct formation. NAC has nucleophilic character and may directly react with electrophilic agents (30). Therefore, NAC may chemically react with AzGalp, decreasing its cytotoxicity.
We next explored if the inhibition of glycolysis played a role in the selective cytotoxicity of AzGalp. During carcinogenesis, cancer cells suffer a metabolic reprogramming, including a high dependence on glycolysis. This metabolic pathway is necessary to obtain energy and metabolic intermediates for macromolecular biosynthesis to support the high proliferation rate of cancer cells (31–33). It is thought that the inhibition of glycolysis could selectively kill cancer cells because they would not support a metabolic reprogramming (22,23). Because sugar analogues, such as 2-deoxy-D-glucose and 2-deoxy-D-galactose, are potent glycolytic inhibitors (22,34) and AzGalp is a β-D-galacto-pyranoside derivative, we tested if this compound could inhibit the glycolysis as a possible mechanism of its selective cytotoxicity. However, the treatment with AzGalp did not decrease neither the consumption of glucose (the initial product of glycolysis) nor the production of lactate (the final product of glycolysis); these data indicate that the cytotoxicity of our aziridine is not mediated by an inhibition of the glycolytic pathway.

Since most anticancer drugs are generally used in combination, we evaluated the effects of the combination of AzGalp with other anticancer agents. Because alkylating agents and antimetabolites are widely used in cancer therapy, we evaluated the cytotoxic effect of the combination of AzGalp with the alkylating agent oxaliplatin and the antimetabolite 5-FU. We observed that the combination of AzGalp with these chemotherapeutic drugs killed more cancer cells and fewer normal cells than any of these drugs individually. This selectivity improvement was mild, however.

We previously reported that AzGalp displayed selective cytotoxicity against lung cancer, breast cancer and melanoma cells (4). In this study, we showed that AzGalp displayed also selective cytotoxic activity against other solid cancer cell lines, such as liver cancer HepG2 cells and osteosarcoma U2OS cells, and against hematological malignancies, such as acute promyelocytic leukemia HL60 and NB4 cells. We compared the cytotoxic activity of AzGalp with the anticancer drug 5-FU. AzGalp displayed better selective cytotoxic profile against acute promyelocytic leukemia cell lines than 5-FU. At concentrations between 10-100 µM, the viability of leukemia cells exposed to AzGalp was reduced progressively until 5%, whereas the viability of the cells exposed to 5-FU remained higher than 20%. At the same range of concentrations, AzGalp did not altered the viability of normal cells, whereas 5-FU displayed cytotoxicity against these cells.

Finally, we used three genetically modified skin cell lines with increasing degree of malignancy to evaluate the selectivity of AzGalp. These cell lines are derived from normal BJ cells, which are altered in specific genes/proteins to progressively acquire the properties of cancer cells (11). AzGalp was selectively cytotoxic to BJ-SV40T and BJ-RASV12 cells (Figure 6), suggesting that this aziridine compound has selective activity against malignant transformed cells. The malignant BJ-RASV12 cells express the oncogene HRAS and have inactive p53 and RB, two major tumor suppressors. The activation of oncogenes and the inactivation of tumor suppressors are frequently found in tumor cells and have been associated to the high basal DNA damage levels of cancer (18,35,36). Besides, cancer cells usually have defects in DNA repair pathways that also contribute to their genomic instability. The high genomic instability and DNA repair defects in tumor cells make them more vulnerable than healthy cells to DNA-damaging agents. Unlike cancer cells, healthy cells have an operative DNA damage response that would allow them to repair the DNA damage and to survive exposition to these DNA-damaging drugs. This could explain why several DNA-damaging drugs used currently in clinic have moderate selective anticancer activity and prolong survival of cancer patient. These data support the idea that the selective cytotoxicity of AzGalp against malignant cells could be mediated by its ability to induce DNA damage.

In conclusion, this article shows that the aziridine compound AzGalp has selective anticancer activity in vitro that is mediated, at least in part, by the induction of DNA damage. It also shows that NER-deficient cells are significantly hypersensitive to the cytotoxicity of AzGalp; this suggests that cancer cells with deficiency in this DNA repair pathway could be hypersensitive to this compound. Our results suggest that AzGalp could have anticancer therapeutic potential because it displayed higher cytotoxicity against several types of cancer cells than against non-malignant cells. Animal studies are necessary to test the efficacy and safety of AzGalp. This aziridine β-D-galactopyranoside derivative could also be a promising lead compound for the development of new anticancer drugs.
5. CONFLICTS OF INTEREST

The authors declare no conflict of interest.

6. REFERENCES


Figure 1. Chemical structure of several aziridine-containing compounds: Thiotepa, mitomycin c, aziridinium intermediates from activation of nitrogen mustard and 2-Methyl-2,3-[N-(4-methylbenzenesulfonyl)imino]propyl 2,3-di-O-benzyl-4,6-O-(S)-benzylidene-β-D-galactopyranoside (AzGalp).
Figure 2. Determination of AzGalp-induced DNA damage in AA8 cells by the comet assay. A) Representative photographs of control cells and cells exposed to 50 µM AzGalp or 10 µM camptothecin (CPT) for 4 h. B) Quantification of DNA damage expressed as percentage of DNA damage in Tail and as Tail Moment (Tail length x percentage of DNA in the Tail). C) Distribution of cells in the different intervals of values of Tail Moments. Data are averaged from two independent experiments.
Figure 3. Cells deficient in nucleotide excision repair (NER) are hypersensitive to the cytotoxicity of AzGalp. A) AA8 cells (NER proficient), UV5 (mutated in XPD; deficient in NER repair) and UV4 (mutated in ERCC1; deficient in NER repair) were treated with several concentrations of AzGalp for 24 h and, after a recovery period of 48 h, cell viability was determined with the MTT assay. B) AA8 and UV4 cells were treated with AzGalp or etoposide (positive control) for 24 h. Then, cells were allowed to form colonies in drug-free medium for 7 days. Finally, the percentage of cell survival with respect to untreated cells was determined by the clonogenic assay. Student's t-test was carried out to compare the cytotoxicity of a particular concentration of the compound between AA8 and UV4 (asterisks), or AA8 and UV5 (hashes).
Figure 4. Neither the generation of reactive oxygen species nor the inhibition of glycolysis participate in the cytotoxicity of AzGalp. A549 cells were treated with AzGalp or H₂O₂ for 48 h in the absence or presence of the antioxidants catalase (A), superoxide dismutase mimetic MnTMPyP (B) and NAC (C). The antioxidants were added 1 hour before AzGalp or H₂O₂. Cell viability was determined by the MTT assay. (D) Percentage of glucose consumed and percentage of lactate produced by A549 cells exposed for 8 h to AzGalp or Dichloroacetate in relation to untreated cells.
Figure 5. Cytotoxicity of AzGalp in combination with the anticancer drugs 5-fluorouracil (5-FU) and oxaliplatin. A549 lung cancer cells and MRC-5 lung normal cells were treated for 48 h with AzGalp alone or in combination with 5-FU or oxaliplatin. Cell viability was assessed by the MTT assay.
Figure 6. Evaluation of cytotoxic activity of AzGalp against five cancer cell lines from solid tumors (HeLa, HepG2, PC3, SW480 and U2OS), two acute promyelocytic leukemia cell lines (HL-60 and NB4), two normal cell lines (MRC-5 and VH10) (A) and three genetically modified cell lines (BJ-hTERT, BJ-SV40T and BJ-RASV12) (C). 5-FU was used as positive control (B and D). Cells were exposed to several concentrations of AzGalp or 5-FU for 72 h and cell viability was determined with the resazurin assay.
Table 1. IC50 values of AzGalp and 5-FU against human non-malignant cell lines and human cancer cell lines. Cells were exposed to the drugs for 72 hours and cell viability was then determined with the resazurin assay.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (Mean ± SEM, µM)</th>
<th>AzGalp</th>
<th>5-FU</th>
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</thead>
<tbody>
<tr>
<td><strong>Normal cells</strong></td>
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<tr>
<td>MRC-5 (Human lung non-malignant cells)</td>
<td>341.1 ± 85.0</td>
<td>&gt; 1000</td>
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<tr>
<td>VH10 (Human skin non-malignant cells)</td>
<td>410.9 ± 39.4</td>
<td>&gt; 1000</td>
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<tr>
<td><strong>Cancer cells</strong></td>
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<tr>
<td>HeLa (Human cervical carcinoma cells)</td>
<td>191.0 ± 35.8</td>
<td>66.3 ± 27.0</td>
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<tr>
<td>HepG2 (Human hepatocellular carcinoma cells)</td>
<td>121.7 ± 12.4</td>
<td>0.6 ± 0.3</td>
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<tr>
<td>HL-60 (Human acute promyelocytic leukemia cells)</td>
<td>11.1 ± 0.9</td>
<td>0.9 ± 0.8</td>
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<tr>
<td>NB4 (Human acute promyelocytic leukemia cells)</td>
<td>21.4 ± 9.9</td>
<td>2.5 ± 0.3</td>
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<td>PC3 (Human prostate cancer cells)</td>
<td>400.7 ± 14.8</td>
<td>1.5 ± 0.3</td>
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<td>SW480 (Human colon adenocarcinoma cells)</td>
<td>387.8 ± 30.9</td>
<td>2.9 ± 1.3</td>
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<tr>
<td>U2OS (Human osteosarcoma cells)</td>
<td>120.5 ± 8.0</td>
<td>6.4 ± 0.7</td>
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<tr>
<td><strong>Genetically modified cells</strong></td>
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<tr>
<td>BJ-hTERT (hTERT-immortalized skin non-malignant BJ cells)</td>
<td>208.7 ± 24.2</td>
<td>2.4 ± 0.5</td>
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<tr>
<td>BJ-SV40T (SV40T-transformed BJ-hTERT cells)</td>
<td>142.7 ± 8.1</td>
<td>5.4 ± 0.7</td>
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<tr>
<td>BJ-RASV12 (HRASV12-transformed BJ-SV40T cells)</td>
<td>76.3 ± 17.8</td>
<td>3.0 ± 1.4</td>
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</table>