# Selective Cytotoxic Activity and DNA Damage by an Epoxyalkyl Galactopyranoside

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**ABSTRACT** Several clinically useful anticancer drugs selectively kill cancer cells by inducing DNA damage; the genomic instability and DNA repair defects of cancer cells make them more vulnerable than normal cells to the cytotoxicity of DNA-damaging agents. Because epoxide-containing compounds can induce DNA damage, we have used the MTT assay to evaluate the selective cytotoxicity of three epoxyalkyl galactopyranosides against A549 lung cancer cells and MRC-5 lung normal cells. Compound (2*S*,3*S*)-2,3-Epoxydecyl 4,6-*O*-(*S*)-benzylidene-β-D-galactopyranoside (EDBGP) showed the highest selective anticancer activity and was selected for mechanistic studies. After observing that EDBGP induced cellular DNA damage (comet assay), we found that cells deficient in nucleotide excision repair were hypersensitive to the cytotoxicity of this compound; this suggests that EDBGP may induce bulky DNA adducts. EDBGP did not inhibit glycolysis (glucose consumption and lactate production). Pre-treatment of lung cancer cells with several antioxidants did not reduce the cytotoxicity of EDBGP, thereby indicating that reactive oxygen species do not participate in the anticancer activity of this compound. Finally, EDBGP was screened against a panel of cancer cells and normal cells from several tissues, including three genetically modified skin fibroblasts with increasing degree of malignancy. Our results suggest that epoxyalkyl galactopyranosides are promising lead compounds for the development of new anticancer agents.

**KEYWORDS:** epoxide, cancer, anticancer, cytotoxicity, nucleotide excision repair

## 1. INTRODUCTION

Cancer is the second leading cause of death in the developed countries (1–3). An important percentage of patients are diagnosed when cells from a primary tumor have already metastasized to other parts of the body. At this stage of the disease, cancer cells are no longer localized and cannot be eliminated by surgery or radiotherapy. The main form of treatment at this point is pharmacotherapy, which consists of delivering drugs systemically so that they can reach and kill the tumor cells. However, most of these drugs are toxic to both cancer and healthy cells, causing

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side effects that can even threaten patient's life. As a consequence, anticancer drugs are generally used at suboptimal doses, which are insufficient to eliminate all the cancer cells (4). Despite this limitation, pharmacotherapy is crucial to delay disease progression when local therapies are no longer indicated.

DNA-damaging drugs (such as alkylating agents, cytotoxic antibiotics and DNA topoisomerase poisons) are widely used as first-line therapy for many types of cancers. Some of these anticancer drugs show moderate selectivity against cancer cells. For a long time, there has not been an adequate explanation for this selectivity (5). It is now accepted that the selective anticancer activity of these cytotoxic drugs is due to the high levels of genetic instability and defects in DNA repair pathways of cancer cells (6,7). The study of the mechanisms involved in the selective anticancer activity of DNA-damaging compounds can lead to better anticancer therapies (6,8).

Alkylating agents are widely used anticancer drugs. Many therapeutically useful alkylating drugs are electrophiles that react with nucleophilic moieties of DNA bases, generating adducts and, subsequently, DNA damage. This is the mechanism of action of the drug treosulfan, an alkylating agent used for ovarian cancer in several European countries (9,10). Treosulfan is a prodrug that is converted non-enzymatically to two biologically active epoxides, which are the responsible of the DNA alkylation and the interstrand cross-linking of DNA (11). The epoxide moiety of these compounds reacts with the nucleophilic groups of DNA, like the DNA base guanine. Other epoxides can also induce DNA damage (12–15). Therefore, compounds containing epoxides in their structure could induce DNA damage. Because cancer cells are more susceptible than normal cells to DNA-damaging agents, we studied the possible selective anticancer activity of several epoxyalkyl galactopyranosides whose synthesis has been described previously (16). The compounds evaluated were (2*R*)-2,3-Epoxypropyl 4,6-*O*-(*S*)-benzylidene-β-D-galactopyranoside (EPBGP), (2*S*,3*S*)-2,3-Epoxydecyl 4,6-*O*-(*S*)-benzylidene-β-D-galactopyranoside (EDBGP) and (2*R*)-2,3-Epoxy-2-methylpropyl 4,6-*O*-(*S*)-benzylidene-β-D-galactopyranoside (EMBGP) (Figure 1). In this work, we report the selective anticancer activity of these epoxides against lung cancer cells versus lung non-malignant cells. We also propose a possible mechanism of action for the most selective compound. Finally, we show its cytotoxic profile in a panel of cancer cells and normal cells from a variety of tissues.

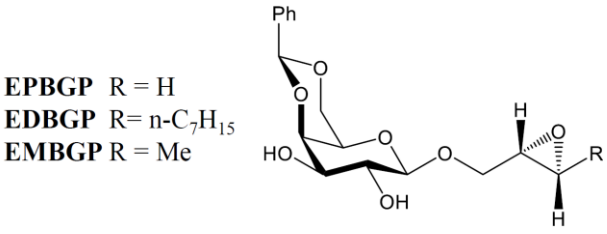


Figure 1. Chemical structure of epoxides tested in this work.

## 2. MATERIALS & METHODS

### 2.1. Chemicals

Cisplatin, 5-fluorouracil (5-FU), camptothecin (CPT), hydrogen peroxide ($H_2O_2$), catalase, N-acetylcysteine (NAC), dichloroacetate (DCA), hydroxyurea, 3-(4,5-dimethylthiazol-2-yl)-2,5-

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diphenyltetrazolium bromide (MTT), resazurin and fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma. Mn(III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) was obtained from Biomol International. The mounting medium for fluorescence (Vectashield) was purchased from Vector Laboratories. (2*R*)-2,3-Epoxypropyl 4,6-*O*-(*S*)-benzylidene-β-D-galactopyranoside (EPBGP), (2*S*,3*S*)-2,3-Epoxydecyl 4,6-*O*-(*S*)-benzylidene-β-D-galactopyranoside (EDBGP) and (2*R*)-2,3-Epoxy-2-methylpropyl 4,6-*O*-(*S*)-benzylidene-β-D-galactopyranoside (EMBGP) were synthesized as described elsewhere (16). All other compounds used in this work were obtained from Sigma. Stock solutions of cisplatin, 5-FU, CPT, hydroxyurea, MnTMPyP and epoxyalkyl galactopyranosides were prepared in DMSO. $H_2O_2$, catalase, NAC, DCA, MTT and resazurin were dissolved in culture medium before use.

### 2.2. Cell lines

A549 (human non-small cell lung cancer cells) and MRC-5 (human fetal lung fibroblastic cells) were purchased from European Collection of Cell Cultures. UACC-62 (human melanoma cells) was obtained from National Cancer Institute (NCI). VH10 (human foreskin fibroblast cells), SW480 (human colon adenocarcinoma cells), HepG2 (human hepatocellular carcinoma cells), U2OS (human osteosarcoma cells), PC3 (human prostate cancer cells), HeLa (human cervical carcinoma cells), NB4 (human acute promyelocytic leukemia cells) and HL-60 (human acute promyelocytic leukemia cells) were generously provided by Dr. Helleday (Karolinska Institute, Sweden) (17). 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) (18). MCF7 (human breast adenocarcinoma cells) and MCF 10 (human mammary epithelial cells) cell lines were a gift from Dr. D. Ruano and Dr. P. Daza.

To study the possible DNA damage response induced by the tested EDBGP, the following parental and DNA repair-deficient cell lines were used: HCT 116+ch3 (HCT 116 cells complemented with chromosome 3; MMR-proficient), HCT 116 (human colon cancer cells mutated in MLH1, mismatch repair (MMR)-deficient), EM9-XH (EM9 cells complemented with XRCC1, BER-proficient), EM9-V (AA8 cells mutated in XRCC1 (DNA ligase III), base excision repair (BER)-deficient), VC8-B2 (VC8 cells complemented with human BRCA2, homologous recombination (HR)-proficient), VC8 (V79 Chinese hamster lung cells mutated in BRCA2, HR-deficient), AA8 (parental Chinese hamster ovary cells, DNA repair proficient), V3-3 (AA8 cells mutated in XRCC7 (DNA-PK), non-homologous end joining (NHEJ)-deficient), UV4 (AA8 cells mutated in ERCC1, nucleotide excision repair (NER)-deficient) and UV5 (AA8 cells mutated in ERCC2 (XPD), NER-deficient). HCT 116+ch3, HCT 116, VC8-B2, VC8, AA8, V3-3, UV4 and UV5 were a gift from Dr Helleday (Karolinska Institutet, Stockholm, Sweden) (19,20). EM9-XH and EM9-V were kindly provided by Dr. Caldecott (University of Sussex, United Kingdom) (21). MRC-5, VH10, A549, MCF7, UACC-62, SW480, HepG2, U2OS, HeLa, BJ-hTERT, BJ-SV40T, BJ-RASV12, VC8-B2, VC8, EM9-XH, EM9-V, AA8, V3-3, UV4 and UV5 were maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose medium. PC3 was grown in DMEM-F12. HL60 and NB4 were maintained as a suspension culture in RPMI 1640. HCT 116+ch3 and HCT 116 were cultured in McCoy's 5A. EM9-XH and EM9-V cells were cultured in the presence of geneticin (G418) at a final concentration of 1.5 mg/ml. All media were supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. MCF 10 was cultured in a 1:1 mixture of Ham's F12 medium and DMEM supplemented with 100 ng/mL cholera toxin, 20 ng/mL epidermal growth factor, 10 mg/mL insulin and 500 ng/mL

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of hydrocortisone (95%) and 5% horse serum. All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2. Cell culture reagents were purchased from Thermo Fisher Scientific.

### 2.3. Cell viability assays

Exponentially growing cells were seeded in 96-well plates and were allowed to grow during 24 h. The cells were then exposed to several concentrations of the tested compounds. After the treatment period, cell viability was determined with the MTT assay or the resazurin assay, two techniques that are widely used to estimate cell viability.

The MTT assay is based on the reduction of yellow tetrazolium MTT by metabolically active cells to an insoluble and purple colored formazan product; dead cells are metabolically inactive and cannot reduce the MTT. The insoluble formazan product is analyzed spectrophotometrically after previous solubilization. 24 hours after seeding, cells were exposed to the tested compounds for 48 h; except in the experiments with the DNA repair-proficient and –deficient cell lines, in which cells were treated for 24 h and were then 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) were added to each well. The plates were incubated for 2-4 hours to allow viable cells to transform the yellow MTT compound into an insoluble formazan product. Then, 80 µL 20% SDS in 20 mM HCl were added to dissolve the insoluble purple formazan product and plates were incubated overnight at 37 °C. Finally, optical densities were measured at 540 nm using an absorbance spectrophotometer microplate reader.

The resazurin assay is a redox-based fluorometric/colorimetric technique based on the reduction of the blue compound resazurin by viable cells into the pink, fluorescent and soluble product resorufin. The amount of resorufin produced is proportional to the number of living cells. 24 hours after plating, cells were treated with 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 read 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 averaged from at least three independent experiments and were expressed as the means ± standard error of the mean (SEM). Since selectivity is a crucial parameter to evaluate anticancer potential in vitro, selectivity indices were calculated (4,22). The selectivity index (S.I.) is calculated as the mean of the IC50 value in the normal cell lines divided by the IC50 value in the cancer cell lines obtained in each independent experiment.

### 2.4. Comet assay

The Comet assay, also known as single cell gel electrophoresis assay, is one of the most commonly used methods for the detection of DNA damage in individual cells. In this assay, isolated cells embedded in agarose are lysed, electrophoresed, stained with a fluorescent dye and examined under epifluorescence microscope. The images obtained with this test resemble a “comet” with a distinct head (undamaged DNA nucleoid part) and tail (single-strand or double-strand breaks). The amount of DNA in the tail indicates the level of DNA damage. This technique has been described in detail by Singh et al (23). We followed this protocol with minor modifications described previously (24). Briefly, standard slides were immersed in 1% normal melting agarose at 55 °C, left to allow the agarose to solidify at room temperature, and kept at 4 °C until use. A549 cells were seeded in 6-well plates and were allowed to grow during 24 h. Then,

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cells were treated with EDBGP or CPT for 4 h. After treatment, cells were harvested by trypsinization, washed with PBS and resuspended in PBS. Approximately $10^4$ cells were mixed with 85 µL of low-melting agarose (LMA) at 37°C and the mixture was rapidly pipetted onto 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 a third 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 4ºC in a lysis buffer (pH 10.0) containing 2.5 M NaCl, 100 mM Na2-EDTA, 0.25 M NaOH, 1% (v/v), Triton X-100, 10% (v/v) DMSO and 10 mM Tris-HCl. To alkaline denaturation of the DNA, the slides were incubated for 20 min in an electrophoretic buffer (300 mM NaOH and 1 mM Na2-EDTA); 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). Finally, cells were stained with DAPI in Vectashield and images were taken with an epifluorescence microscope. A total of approximately 50 cells from each sample were analyzed using the CometScore software. DNA damage was calculated for each comet 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 from two independent experiments and were expressed as mean ± SEM.

### 2.5. 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. $10^6$ cells were seeded into 6-well plates and were allowed to attach 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 (25). Results are expressed as percentage of glucose consumption and percentage of lactate production in relation to untreated cells. Data are shown as the mean ± SEM of two independent experiments.

### 2.6. 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. Evaluation of selective cytotoxic activity of epoxyalkyl galactopyranosydes against A549 lung cancer cells and MRC-5 lung normal cells

We initially tested the anticancer activity of these epoxides on a lung cancer model *in vitro*. We exposed A549 human lung cancer cells and MRC-5 human non-malignant lung fibroblast to three epoxyalkyl galactopyranosides and the anticancer drug cisplatin for 48 h. The results are represented in Figure 2 and Table 1. A549 cancer cells were more sensitive to the cytotoxic activity of these epoxide-containing compounds than MRC-5 normal cells. EPBGP showed a very

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slight selectivity against cancer cells; the IC50 values in the A549 cancer cells was 1.4 times lower than in the MRC-5 normal cells. EDBGP and EMBGP showed a relevant selective cytotoxic activity, especially EDBGP whose IC50 value in A549 was approximately 3-fold lower than in MRC-5. The selectivity index values for EPBGP, EDBGP, EMBGP and cisplatin were 1.4, 2.9, 2.1 and 12.8, respectively. Since EDBGP showed the most potent and selective activity, our next aim was to evaluate possible mechanisms involved in its cytotoxic activity.

Table 1. Evaluation of selective anticancer activity of epoxyalkyl galactopyranosides and cisplatin against A549 lung cancer cells and MRC-5 lung normal cells. Cells were treated for 48 hours and cell viability was estimated with the MTT assay.

| **Cell Lines** | **IC50 (Mean ± SEM, µM)** | | | |
|---|---|---|---|---|
| | **EPBGP** | **EDBGP** | **EMBGP** | **Cisplatin** |
| **MRC-5** (Lung normal cells) | 556.3 ± 24.7 | 209.1 ± 54.2 | 334.1 ± 22.3 | 140.8 ± 50.4 |
| **A549** (Lung cancer cells) | 405.1 ± 38.8 | 75.4 ± 25.3 | 160.9 ± 1.2 | 12.6 ± 4.8 |

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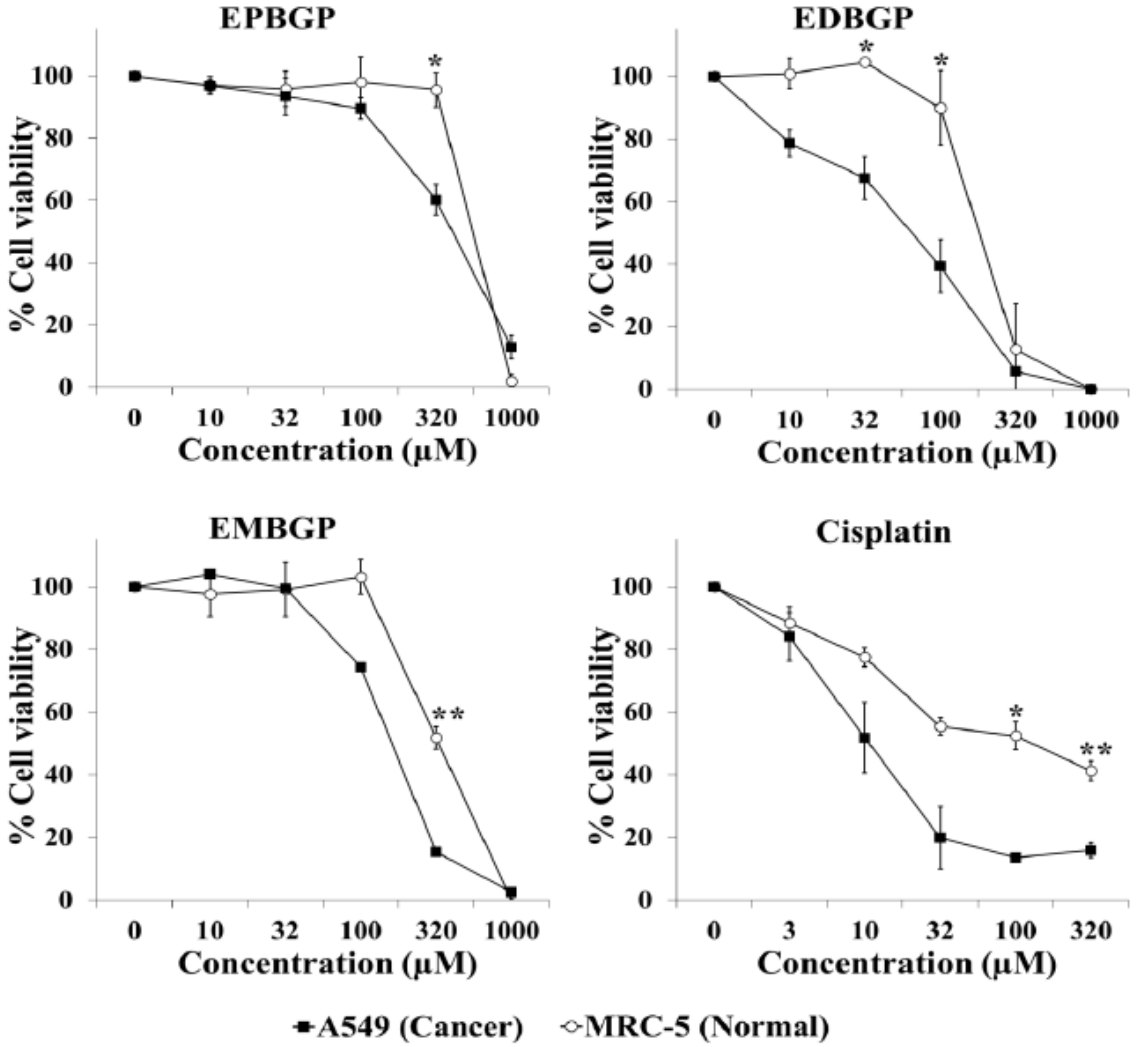


Figure 2. Cytotoxic activity of epoxyalkyl galactopyranosides and cisplatin on A549 lung cancer cells and MRC5 lung normal cells. Cells were exposed to the compounds for 48 h and cell viability was then estimated with the MTT assay.

### 3.2. EDBGP induces DNA damage

Compounds that contain an epoxide in their chemical structure can induce DNA damage (12–15), so we hypothesized that EDBGP could induce DNA damage. To test this hypothesis, we employed a standard method for DNA damage detection: the comet assay (23). Camptothecin (CPT), a standard DNA-damaging agent, was used as positive control. Figure 3 shows representative photographs of cells exposed to EDBGP or CPT for 4 h, the quantification of DNA damage and the percentage of cells within different ranges of DNA damage. We observed that cells treated with 300 µM EDBGP had higher levels of DNA in the tail of the "comets" than untreated cells, indicating that EDBGP induces DNA damage. The levels of DNA damage were similar to those induced by camptothecin.

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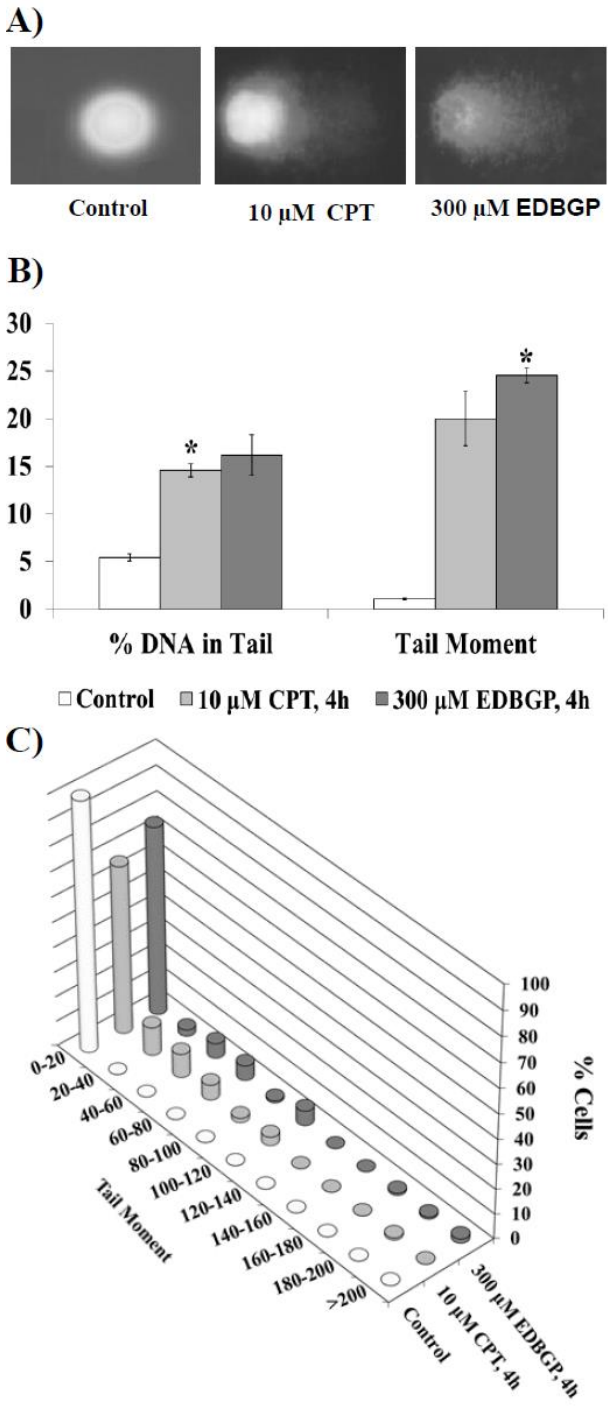


Figure 3. Assessment of EDBGP-induced DNA damage in A549 lung cancer cells by the comet assay. A) Representative photographs of untreated cells (control), of cells treated with the positive control camptothecin (CPT), and of cells exposed to EDBGP for 4 hours. B) Quantification of DNA damage expressed 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. Results are averaged from two independent experiments.

### 3.3. Nucleotide excision repair-deficient cells are sensitive to the cytotoxic effect of EDBGP

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Next, we used a panel of cell lines deficient in several DNA repair pathways to elucidate the type of DNA damage induced by EDBGP. All cell lines were exposed to several concentrations of EDBGP for 24 h and, after a recovery time of 48 h, cell viability was determined with the MTT assay. IC50 values were calculated for each cell line and are represented in Table 2. The cytotoxicity of EDBGP was not affected by defects in mismatch repair, base excision repair, homologous recombination and non-homologous end joining. However, Figure 4 shows that this compound was approximately 2.4–fold more cytotoxic against cells deficient in nucleotide excision repair (UV4 and UV5 cells) than against their parental cell line (AA8, NER proficient cell). These data suggest that the DNA damage induced by EDBGP participates in its cytotoxic activity and requires a functional NER for repair.

Table 2. Cytotoxic activity of EDBGP on a panel of DNA repair deficient cell lines. Cells were exposed to EDBGP for 24 h and, after a recovery period of 48 h, cell viability was determined by the MTT assay.

| **Cell lines** | **IC50 (Mean ± SEM, µM)** |
|---|---|
| **HCT 116+ch3** (MLH1 complemented, MMR-proficient) | 64.7 ± 0.2 |
| **HCT 116** (MLH1 mutant, MMR-deficient) | 43.5 ± 16.1 |
| **EM9-XH** (XRCC1 complemented, BER-proficient) | 160.0 ± 18.8 |
| **EM9-V** (XRCC1 mutant, BER-deficient) | 191.4 ± 9.8 |
| **VC8-B2** (BRCA2 complemented, HR-proficient) | 113.3 ± 31.7 |
| **VC8** (BRCA2 mutant, HR-deficient) | 114.1 ± 29.0 |
| **AA8** (Wild-type) | 174.9 ± 18.7 |
| **V3-3** (DNA-PKcs mutant, NHEJ-deficient) | 125.4 ± 35.7 |
| **UV4** (ERCC1 mutant, NER-deficient) | 107.2 ± 50.9 |
| **UV5** (XPD mutant, NER-deficient) | 92.6 ± 32.8 |

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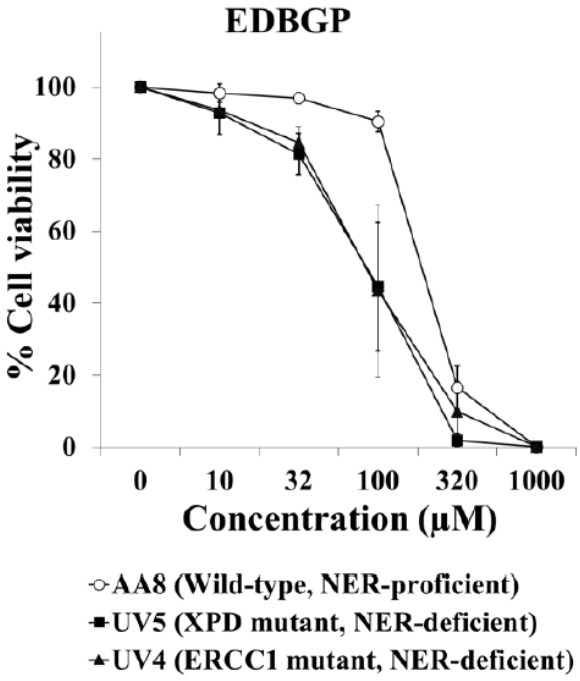


Figure 4. Nucleotide excision repair (NER)-deficient cells are sensitive to EDBGP. AA8 cells (NER proficient), UV5 (mutated in XPD; deficient in NER repair) and UV4 (mutated in ERCC1; deficient in NER repair) were exposed to several concentrations of EDBGP for 24 h. After treatment, cells were allowed to recover for 48 h and cell viability was measured with the MTT assay.

### 3.4. Study of other mechanisms involved in the EDBGP-induced cytotoxicity.

Our next goal was to evaluate other possible mechanisms involved in the selective cytotoxicity of this compound. The generation of ROS is known to play an important role in the cytotoxic activity of several current anticancer drugs (26–29). We evaluated whether the formation of ROS was involved in the cytotoxicity of EDBGP. We tested the cytotoxic activity of the EDBGP in the presence or absence of the antioxidants catalase (Figure 5A), MnTMPyP (Figure 5B) or N-acetylcysteine (Figure 5C), and the cell viability was determined by the MTT assay. The incubation with the three antioxidants reduced the cytotoxicity induced by hydrogen peroxide, used as positive control. However, treatment with these antioxidants did not alter the cytotoxicity of EDBGP. These results suggest that the cytotoxic activity of EDBGP is not mediated by the generation of ROS.

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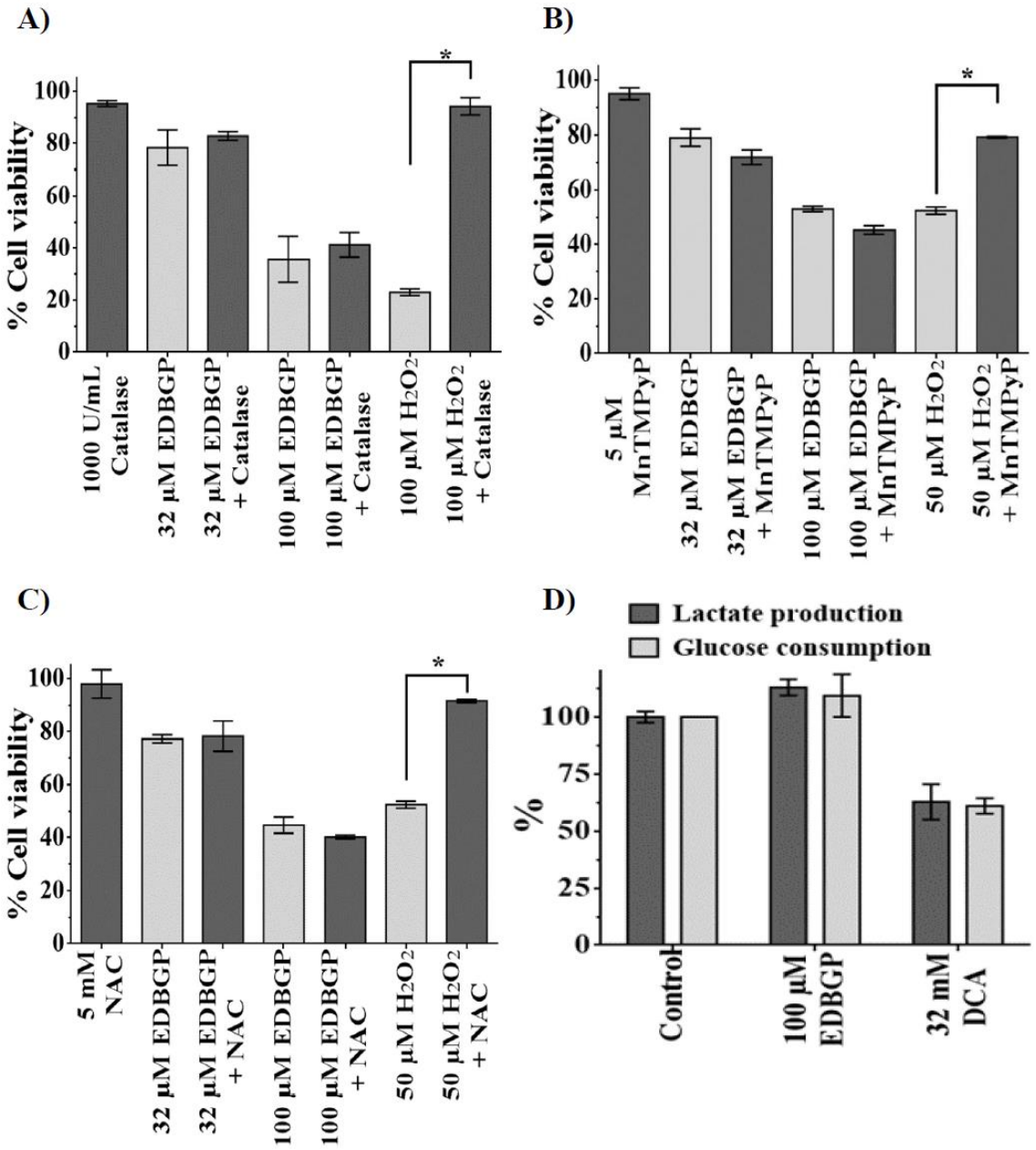


Figure 5. Neither the generation of reactive oxygen species nor the inhibition glycolysis are involved in the cytotoxic activity of EDBGP. A549 cells were exposed to EDBGP or $H_2O_2$ for 48 h in the absence or presence of the antioxidants catalase (A), the superoxide dismutase mimetic MnTMPyP (B) and NAC (C). The antioxidants were added 1 hour before EDBGP or $H_2O_2$. After treament, cell viability was determined with the MTT assay. (D) Percentage of lactate produced and percentage of glucose consumed by A549 cells exposed for 8 h to EDBGP or Dichloroacetate (DCA) in relation to untreated cells.

We next studied whether the inhibition of glycolysis played a role in the cytotoxicity of EDBGP. Accumulating data suggest that tumor cells have much higher levels of glucose intake and glycolysis activity than normal cells (Warburg effect) (30,31), and that glycolysis inhibition may induce selective anticancer effects (32,33). The possible inhibition of glycolysis by EDBGP was evaluated by measuring concentrations of glucose consumed (initial product of glycolysis) and lactate produced (final product of glycolysis) in untreated and treated A549 cancer cell line. Cells

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were treated for 8 h, time enough to detect alterations in the glycolytic rate but without causing a reduction in cell number (fewer cells would consume less glucose and would produce less lactate). The glycolysis inhibitor DCA, used as control positive, reduced the glucose consumption and the lactate production of cells treated. There was no difference between untreated cells and cells treated with EDBGP. Therefore, our results show that glycolysis is not altered in the presence of our epoxide (Figure 5D).

### 3.5. Evaluation of the cytotoxic activity of EDBGP against a panel of cancer cells and non-malignant cells

Our next aim was to assess the selective cytotoxic activity of EDBGP in other cancer cell lines. We determined the selective cytotoxic activity against breast cancer, melanoma and colon cancer cell lines. Cancer cells (MCF7, UACC-62 and HCT 116) and non-malignant cells (MCF 10 and VH10) were exposed to several concentrations of the EDBGP or anticancer drugs (5-fluorouracil and hydroxyurea) for 48 h, and cell viability was estimated by the MTT assay. Results are represented in Figure 6 and Table 3. The MCF7 human breast cancer cells were 2.8-fold more sensitive to EDBGP than the MCF 10 breast normal cells. The UACC-62 human melanoma cells were 2.4 times more sensitive than the human skin normal cells VH10. We also observed that this epoxide showed cytotoxic activity against HCT 116 human colorectal carcinoma cells similar to MCF7 breast cancer cells. It is worth mentioning that EDBGP showed better selective profile against breast cancer cells than the anticancer drug 5-fluorouracil and similar selectivity against melanoma cells than the anticancer compound hydroxyurea.

Table 3. Evaluation of selective anticancer activity of EDBGP, 5-FU and hydroxyurea against breast, melanoma and colorectal cancer cells. Cells were treated for 48 hours and cell viability was estimated with the MTT assay.

| **Cell Lines** | **IC50 (Mean ± SEM, µM)** | | |
|---|---|---|---|
| | **EDBGP** | **5-FU** | **Hydroxyurea** |
| **MCF 10** (Breast non-malignant cells) | 111.9 ± 6.1 | < 0.1 | N.d. |
| **MCF7** (Breast cancer cells) | 40.0 ± 1.1 | 0.127 ± 0.003 | N.d. |
| **VH10** (Skin normal cells) | 227.8 ± 62.5 | > 1000 | > 1000 |
| **UACC-62** (Melanoma cells) | 104.6 ± 25.9 | 195.7 ± 190.4 | 521.3 ± 190.3 |
| **HCT 116** (colorectal cancer cells) | 48.2 ± 6.9 | 1.7 ± 0.4 | N.d. |

N.d.: Not determined.

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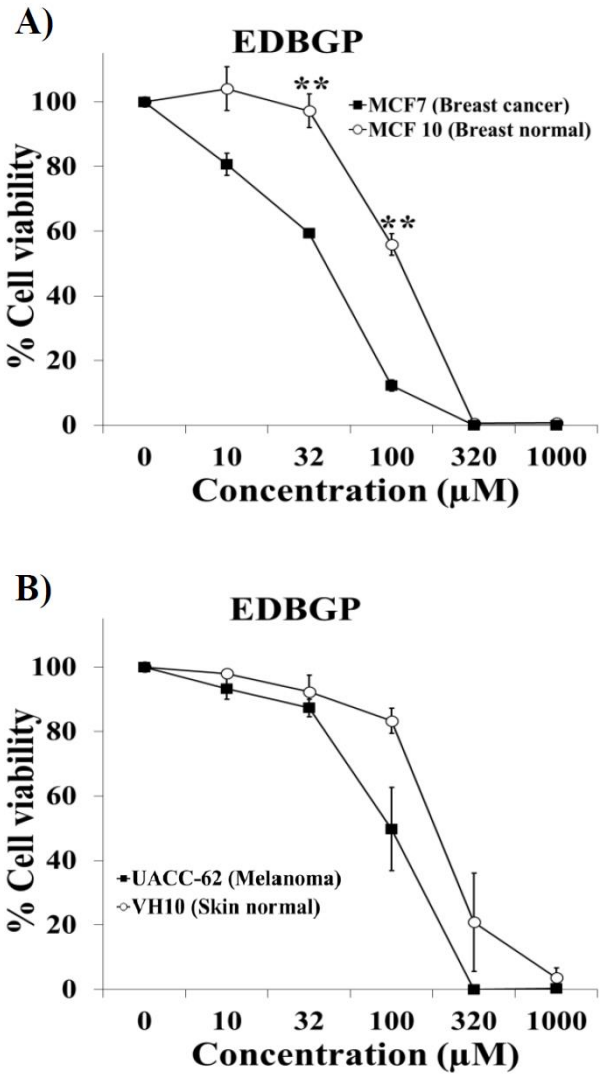


Figure 6. EDBGP has selective cytotoxicity against breast cancer and melanoma cell lines. MCF7 breast cancer cells, MCF 10 breast normal cells (A), UACC-62 melanoma and VH10 skin normal cells (B) were treated with EDBGP for 48 hours. After treatment, cell viability was determined with the MTT assay.

Because EDBGP displayed cytotoxic activity against four different cancer cell lines, we tested if EDBGP could also induce cytotoxicity against other cancer cells from solid tumors and blood cancers. Because some of the cells grew as a suspension culture, we evaluated cell viability with the resazurin assay. Unlike the MTT assay, this technique does not require neither removal of medium nor cell washing. All cells were exposed to the tested drug for 72 h and cell viability was then determined by the resazurin assay. The IC50 values calculated for each cell line are shown in Table 4, and concentration-response curves are represented in Figure 7. EDBGP showed the most cytotoxic effect against HL-60 acute promyelocytic leukemia cells and MCF7 breast cancer cells, while this epoxide induced similar cytotoxicity against HeLa cervical carcinoma cells and SW480 colon adenocarcinoma cells than against VH10 and MRC-5 normal cells.

Table 4. Cytotoxic activity of EDBGP and 5-FU on human cell lines after a 72-h treatment (resazurin assay).

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| Cell line | IC50 (Mean ± SEM, µM) | |
|---|---|---|
| | EDBGP | 5-FU |
| **VH10** (Human skin non-malignant cells) | 189.8 ± 15.5 | > 1000 |
| **MRC-5** (Human lung non-malignant cells) | 360.8 ± 72.0 | > 1000 |
| **A549** (Human lung adenocarcinoma cells) | 124.0 ± 8.7 | 0.7 ± 0.2 |
| **HepG2** (Human hepatocellular carcinoma cells) | 153.8 ± 9.2 | 0.49 ± 0.02 |
| **SW480** (Human colon adenocarcinoma cells) | 232.2 ± 29.1 | 1.7 ± 0.7 |
| **U2OS** (Human osteosarcoma cells) | 193.1 ± 8.0 | 6.1 ± 1.0 |
| **HeLa** (Human cervical carcinoma cells) | 302.0 ± 57.4 | 39.4 ± 0.8 |
| **PC3** (Human prostate cancer cells) | 148.8 ± 14.0 | 1.3 ± 0.6 |
| **MCF7** (Human breast adenocarcinoma cells) | 99.7 ± 27.2 | 1.2 ± 0.7 |
| **NB4** (Human acute promyelocytic leukemia cells) | 170.9 ± 18.6 | 2.3 ± 0.2 |
| **HL-60** (Human acute promyelocytic leukemia cells) | 80.9 ± 16.0 | 0.12 ± 0.06 |
| **BJ-hTERT** (hTERT-immortalized skin non-malignant BJ cells) | 367.1 ± 52.1 | 2.1 ± 0.4 |
| **BJ-SV40T** (SV40T-transformed BJ-hTERT cells) | 226.3 ± 26.6 | 4.8 ± 0.6 |
| **BJ-RASV12** (HRASV12-transformed BJ-SV40T cells) | 262.2 ± 31.0 | 3.8 ± 3.0 |

We also tested this compound in three genetically modified cell lines (BJ-hTERT, BJ-SV40T and BJ-RASV12). BJ-hTERT cells are normal foreskin BJ with an active telomerase expression to prevent senescence, BJ-SV40T cells are BJ-hTERT with inactivated p53 and RB (two tumor suppressors), and BJ-RASV12 cells are BJ-SV40T cells with an active HRASV12 expression which is associated with numerous carcinogenic events, such as DNA damage and DNA replicative stress. BJ-hTERT cells are considered as non-tumorigenic, BJ-SV40T as pre-tumorigenic and BJ-RASV12 as tumorigenic cells. EDBGP showed modest selective cytotoxicity against the tumorigenic BJ-RASV12 cells.

## 4. DISCUSSION

Metastasis will continue to be an incurable disease for most patients until we develop new treatments with a high selectivity against cancer cells. As explained in the introduction section, DNA-damaging agents can induce selective killing of cancer cells, and epoxide-containing compounds can induce DNA damage. We therefore initiated our investigation by screening the selective cytotoxic activity of three epoxyalkyl galactopyranosides previously synthesized in our laboratory (16) against lung cancer cells and lung normal cells. Lung cancer is one of the main cause of mortality in the world (1,2) and there is a clear need to find more tolerable and effective treatments. The three tested epoxides showed selectivity against A549 lung cancer cells, especially EDBGP which was approximately 3-fold more cytotoxic against cancer cells than normal cells. Although this compound displayed a lower selectivity index than the anticancer drug cisplatin, 2.9 and 12.8 respectively, the concentration-response curves (Figure 2) were similar for both compounds.

Our next goal was to study possible mechanisms involved in the selective cytotoxic effect of EDBGP. Epoxide-containing compounds, such as benzo[a]pyrene metabolites and aflatoxins, are potential mutagenic and carcinogenic due to their ability to interact with DNA bases as guanine and form DNA adducts that impair transcription and replication (34–37). This interaction with

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DNA is due to the high reactivity of the epoxide ring toward nucleophilic groups in DNA bases, such as guanine and adenosine (38). However, this reactivity with DNA can also be therapeutically useful. Treosulfan, an anticancer drug employed for the treatment of ovarian cancer, is a prodrug that is converted under physiological conditions to L-diepoxybutane via a monoepoxide intermediate (9,11). Both metabolites are responsible for the cytotoxicity of treosulfan. They alkylate DNA at guanine residues, generating adducts and interstrand crosslinks, resulting in cell death (9,11). The ability of epoxides to interact with DNA bases suggested that EDBGP could also induce DNA damage. Using the comet assay, we observed that EDBGP induced cellular DNA damage (Figure 3).

We next used a panel of cell lines deficient in several DNA repair pathways to elucidate the type of DNA damage induced by EDBGP (Figure 4 and Table 2). Data suggest that mismatch repair, homologous recombination and nonhomologous end joining are not involved in the cytotoxicity of this epoxide. Although evidence suggest DNA damage induced by alkylating agents can generate apurinic sites which can be repaired by the BER pathway, we did not observe higher cytotoxicity of our epoxide against BER-deficient cells. NER-deficient cells were 2.4-fold more sensitive than their parental cell line (NER-proficient). This higher sensitivity under NER deficiency was expected because epoxides are known to induce bulky adducts in the DNA, which are usually repaired by the NER pathway (15,35,37,39,40). NER repairs a wide range of DNA helix-distorting lesions that alter transcription and replication, including DNA damage induced by classic alkylating agents (7). Our results suggest that EDBGP may induce bulky distortions in the DNA and that are repaired by NER. Evidence suggests that some lung cancers are deficient in NER (41); these cancers may be hypersensitive to the cytotoxic effect of this epoxyalkyl compound.

We performed several assays to evaluate the possible involvement of other mechanisms of action in the selective cytotoxic activity of EDBGP. Because reactive oxygen species (ROS) have an important role in cancer, we tested whether the generation of ROS was involved in the cytotoxicity of EDBGP. It is known that ROS can induce oxidative DNA damage, which is involved in the cytotoxicity of anticancer drugs (17,28,42). In addition, evidence show that cancer cells have higher intracellular basal levels of ROS than healthy cells. These higher levels of ROS could make cancer cells more sensitive to pro-oxidant agents or antioxidant-defense inhibitors (29,43). However, pre-treatment of lung cancer cells with several antioxidants did not reduce the cytotoxicity of EDBGP (Figure 5), thereby indicating that ROS do not participate in the anticancer activity of this compound.

Many cancer cells depend on high glycolytic rates for their survival. Glycolysis is the catabolic process that employs glucose to obtain energy and metabolic intermediates for macromolecular biosynthesis. Cancer cells keep sustained glycolytic rates despite the presence of an adequate oxygen supply (Warburg effect) (30,31). To keep the high glucose demand, cancer cells have to overexpress glucose transporters (GLUT) and glycolytic enzymes (44–47). This metabolic difference between cancer and normal cells could be a good target to develop new selective anticancer strategies. One of these strategies consists in inhibiting glycolytic enzymes to force a metabolic reprogramming that cancer cells may not resist and they would die. Sugar analogues (2-deoxy-D-glucose and 2-deoxy-D-galactose) are potent glycolytic inhibitors that have shown to kill cancer cells (31,48). Other drugs such as dichloroacetate or cardiac heterosides have also shown a relevant selectivity against cancer cells due to their ability to inhibit glycolysis (32,33,49). Because EDBGP is a β-D-galacto-pyranoside derivative, we hypothesized this compound could inhibit enzymes involved in glycolysis; the inhibition of glycolysis could explain

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their selectivity towards cancer cells. However, EDBGP did not inhibit glycolysis (glucose consumption and lactate production) in lung cancer cells (Figure 5D), thereby suggesting that glycolysis inhibition does not play a role in its cytotoxicity.

Finally, we tested the selective cytotoxic activity of this epoxide on other common types of cancer, including: breast cancer, melanoma and colon cancer (2). This compound showed different cytotoxic profile depending on the cancer type (see Figures 6-7 and Tables 3-4). MC7 breast cancer cells, UACC-62 melanoma cells and HL-60 acute promyelocytic leukemia cells were the most sensitive to the cytotoxic effect of EDBGP. In addition, the cytotoxicity of this compound was also evaluated against three genetically modified cell lines with different degree of malignancy increased (Figure 7 and Table 4). EDBGP showed modest, but statistically significant, selective cytotoxicity against the most malignant transformed cell line (BJ-RASV12). Ras proteins (HRas, NRas and KRas) are essential to signalling pathways involved in cellular proliferation, differentiation and survival. Mutations/aberrations in RAS genes or components of Ras signalling network are frequently found in human cancers and play an important role in the initiation and development of tumours (50,51). These data suggest that EDBGP has selective cytotoxic activity against malignant cells.

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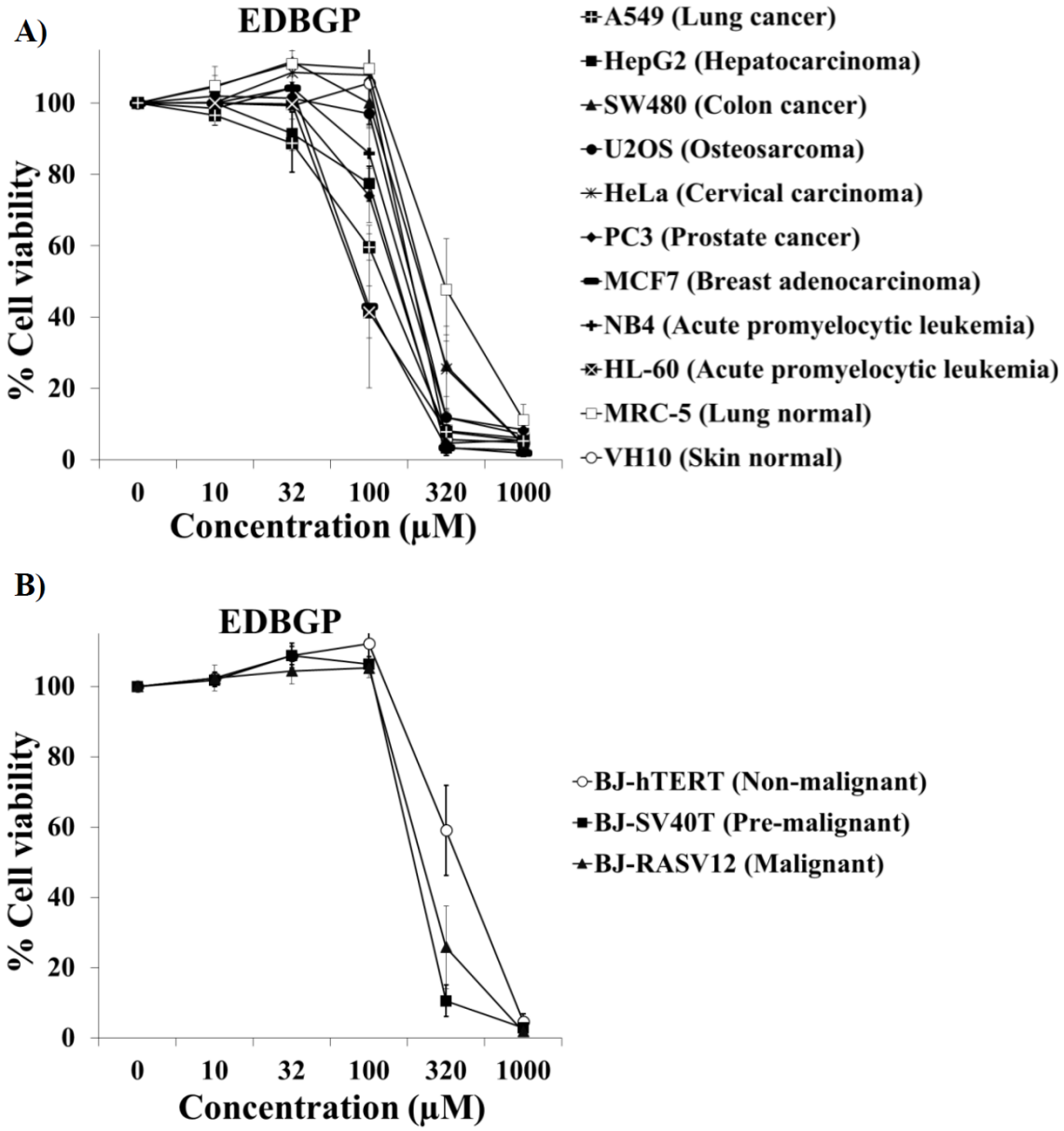


Figure 7. Cytotoxic effect of EDBGP against seven cancer cell lines derived from solid tumors (HeLa, PC3, MCF7, A549, U2OS, HepG2 and SW480), two acute promyelocytic leukemia cell lines (NB4 and HL-60), two normal cell lines (MRC-5 and VH10) (A) and three genetically modified cell lines (BJ-hTERT, BJ-SV40T and BJ-RASV12) (B). Cells were treated for 72 hours and cell viability was estimated with the resazurin assay.

In summary, the epoxyalkyl galactopyranoside (2*S*,3*S*)-2,3-epoxydecyl 4,6-*O*-(*S*)-benzylidene-β-D-galactopyranoside induces selective cytotoxic activity against lung cancer cells, breast cancer cells and melanoma cells. This compound induces DNA damage, which may require a functional NER pathway for repair. Animal models are necessary to test the efficacy and safety of EDBGP in an in vivo setting. Our results also suggest that epoxyalkyl galactopyranosides are promising lead compounds for the development of new anticancer agents.

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