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Possible contribution of β-glucosidase and caspases in the cytotoxicity of glufosfamide in colon cancer cells

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ABSTRACT

Glycoconjugates represent a recent trend in cancer chemotherapy that adopts the concept of selective prodrug/drug targeting of tumor cells by binding to specific transmembrane glucose transporters. Following preferential uptake of sugar conjugates into cancer cells, they are presumably subject to enzymatic cleavage by specific β-glycosidases to liberate the free active cytotoxic aglycones that act selectively on cancer cells and spare other noncancerous ones. In this sense, the role of β-glucosidase and caspases in the bioactivation and cytotoxicity of glufosfamide has been addressed in the current study. The cytotoxicity of glufosfamide has been investigated over 24–96 h in a panel of human colon cancer cells namely, Caco-2, HT29 and T84 using a tetrazole dye; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT assay technique. Apoptosis was assessed by fluorometric assay of caspase-3 and caspase-9 activities. Enzymatic cleavage of glufosfamide was accomplished using a host of hydrolytic enzymes and cleavage kinetics was determined using HPLC. Glufosfamide has proven cytotoxic efficacy in a concentration- and time-dependent manner. The sensitivity rank order of tumor cells towards the glycoconjugate was Caco-2>HT29>T84. This sensitivity ranking was well correlated with the enzymatic activity of β-glucosidase assessed in these cell lines. Initiation and activation of apoptosis were increased in all colon cancer cells following exposure to glufosfamide and were well correlated with the cytotoxicity rank order of the glycoconjugate. Glufosfamide was cleaved by cytosolic and lysosomal β-glucosidases but not by other hydrolytic enzymes such as cytosolic β-galactosidase, pancreatic lipase or hepatic esterase. In conclusion, the current data could possibly unravel the mechanistic role of β-glucosidase and apoptotic caspases in the bioactivation and cytotoxicity of glufosfamide within colon cancer cells.

1. Introduction

The anti-tumor efficacy of clinically used anti-cancer drugs has been almost always hampered by a host of dose-limiting toxicities. Multidrug therapy is a standard modality for the treatment of most cancers. With such intensive chemotherapy, systemic toxicity to the host remains a substantial problem, and cures are achieved only in a small set of cancers (Chari, 2008). The lack of tumor selectivity of anti-cancer drugs and the development of multidrug resistance have given impetus to the development of target-specific new cytotoxic compounds (Wu et al., 2007). Tumor-selective approaches target-specific receptors or other markers that are expressed on the surface of human tumor cells. The targeting molecule can be a monoclonal antibody (Deyev and Lebedenko, 2008), a peptide (Guan et al., 2008), a vitamin (Gupta et al., 2008), a hormone (Ma et al., 2008), an enzyme (Devalapally et al., 2007) or a growth factor, such as an epidermal growth factor (Song et al., 2008).

Amongst the cancer drug targeting modalities recently developed is the concept of saccharides/anti-tumor conjugates; glycoconjugates, i.e., by synthesizing anti-cancer drugs containing sugar moieties that allow the specific binding of the anti-tumor drugs with specific glucose transporters, which transfer the drugs to the tumor cells, where they are selectively uptaken and, thus, minimizing the organ toxicity of these compounds. The rationale behind the development of such glycoconjugates resides for the most part on the glucose avidity of tumor cells based on what is so called the "Warburg effect". Tumor cells exhibit an altered carbohydrate metabolism, characterized by increased glucose uptake and elevated glycolysis, which was first recognized in the 50s by Otto Warburg (Young and Anderson, 2008). Thus, following glycoconjugates entrapment within cancer cells they are presumably subject to hydrolysis by specific β-glycosidases to liberate the active aglycone part that targets the DNA of the cells and exerts its cytotoxic effect. Glufosfamide was the first cytotoxic glycoconjugate to be synthesized by conjugating glucose with ifosfamide mustard (Pohl et al., 1995). Glufosfamide has proven cytotoxic effects in preclinical and clinical trials including non-small cell lung, pancreatic and breast cancers (ADIS R&D PROFILE, 2005). The cytotoxicity of glufosfamide in colon cancer cells has never been tested before. Thus, the cytotoxicity of the glycoconjugate in a panel of colon cancer cells, namely Caco-2, HT29 and T84 was assessed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

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assay. The postulate that enzymatic cleavage by β-glycosidases is crucial for the liberation of free drug aglycones from their respective glycoconjugates was the motive behind this study. Herein, β-glucosidase activities in these cancer colon cells were determined and correlated with the cytotoxicity. Also, enzymatic cleavage by a host of hydrolytic enzymes and cleavage kinetics of glufosfamide were also achieved. There is an emerging realization that cancer chemotherapeutic agents act primarily by inducing cancer cell death through apoptosis (Hannun, 1997). Hence, apoptotic caspases either as initiator (caspase-9) or executioner (caspase-3) were investigated as a mechanistic clue for the cytotoxicity of glufosfamide in these colon cancer cells. Thus, the main objective of the current study was to address the possible contribution of β-glucosidase and caspases in the bioactivation and cytotoxicity of glufosfamide in these tumor cell lines.

2. Materials and methods

2.1. Drugs

Glufosfamide (D-glucose-ifosfamide; β-D-Glc-IPM), was a kind gift from Dr. Bernd Soerg (Molecular Toxicology, Deutsches Krebsforschungszentrum; DKFZ, Heidelberg, Germany).

2.2. Chemicals

P-nitrophenyl-β-D-glucopyranoside was purchased from Fluka (Neu Ulm, Germany). Cytosolic and lysosomal β-glucosidases, cytosolic β-galactosidase, porcine liver esterase, porcine pancreatic lipase, MTT and brilliant blue were obtained from Sigma-Aldrich (Deisenhofen, Germany). Sterile fetal bovine serum (FBS), L-glutamine, trypsin, EDTA, penicillin/streptomycin, RPMI 1640, EMEM, MacCoy's 5a, PBS and HEPES buffer were from Pan Systems (Aidenbach, Germany). Acetonitrile, methanol and bi-distilled water were all of chromatographic grade and obtained from Merck (Darmstadt, Germany). All other chemicals were of the finest analytical grade.

2.3. Cell lines and culture specifications

Three colon cancer cell lines namely; Caco-2, HT29 and T84 were obtained from the Tumor Bank of DKFZ (Heidelberg, Germany). All cell lines grew as monolayer. Caco-2 cell lineage is a human colon adenocarcinoma of epithelial origin isolated from a Caucasian male. Cells were grown in EMEM + 2 mM L-Glutamine + 1% Non Essential Amino acids + 10% FBS. Cells were cultured 1:3 to 1:6; seeding was achieved at $2–4\times10^3$ cells/cm^2. Cells were harvested using trypsin/EDTA and incubated in 5% CO_2 at 37 °C. The hypertetraploid cells were split when sub-confluent. HT29 cell lineage is a human adenocarcinoma grade II of epithelial origin isolated from a Caucasian female. Cells were cultured in McCoy's 5a + 2 mM L-Glutamine + 10% FBS. Cells were cultured 1:3 to 1:10; seeding was done at $1–3\times10^3$ cells/cm^2. Cell harvest was achieved using 0.25% trypsin and cells were incubated in 5% CO_2 at 37 °C. The hypertriploid cells were split when sub-confluent. T84 cell lineage is a transplantable human carcinoma derived from a lung metastasis of a colorectal carcinoma in a 72-year-old male. Subcultivation of 1:2 to 1:4 was achieved. Cells were cultured in RPMI 1640 medium + 2 mM L-glutamine + 10% FBS. Cells were seeded at high density (2.5×10^5 cells/cm^2). Cells were harvested by trypsinization and then incubated in CO_2 at 37 °C. Penicillin (100 units/ml) and Streptomycin (100 μg/ml) were added in all culture flasks.

2.4. MTT cytotoxicity assay

Cytotoxicity of glufosfamide was assessed using MTT technique as earlier described by Weichert et al. (1991). In brief, 100 μl of cell inoculums of each cell line was seeded in their corresponding media per well in 96-well plates. After 72 h, media were removed and replaced by fresh media containing various concentrations of the test drug in the range of 2–64 μM. Each concentration was tested in triplicates. Cell viability was determined after 24, 48, 72 and 96 h. After incubation with the drug, MTT was added to culture wells in a final concentration of 0.5 mg/ml. Cells were incubated for 4 h at 37 °C and the colored formazan product was extracted with 200 μl DMSO and measured at 570 nm using an ELISA reader. Optical density was a direct measure of cell survival. Survival curves were constructed and mean IC_{50} values of glufosfamide at all time points were computed.

2.5. Determination of caspase-9 activity

Caspase-9 activity, one of the initiators of apoptosis, was assessed according to the method earlier described by Li et al. (1997) using a fluorometric kit purchased from R & D Systems (MN, USA). Enzymatic activity following exposure to 5–25 μM glufosfamide for 24 h was assessed as fold increase relative to untreated control cells. Enzyme kinetics was also achieved following exposure to glufosfamide (10 μM) over a time period of 0–24 h. In brief, colon cells were first lyzed with a lysis buffer. Then, cell lysate was tested for protease activity by the addition of a caspase-specific peptide that is conjugated to the fluorescent reporter molecule 7-amino-4-trifluoromethyl coumarin (AFC). The cleavage of the peptide by the caspase releases the fluorochrome that when excited by light at 400 nm wavelength, emits fluorescence at 505 nm. Enzymatic activity in the cell lysate was directly proportional to the fluorescence signal detected with a fluorescent microplate reader (Fluoroskan Ascent, Labsystems, Bornheim-Hersel, Germany).

2.6. Assessment of caspase-3 activity

Caspase-3 activity as the main executioner of apoptosis was measured as described previously by Nicholson et al. (1995) using a commercial fluorometric kit from Calbiochem (Bad Soden, Germany). Enzymatic activity following exposure to 5–25 μM glufosfamide was assessed as fold increase relative to untreated control cells. Also, enzyme kinetics was done using glufosfamide (10 μM) over a time period of 0–24 h. In short, Caco-2, HT-29 and T84 cells were seeded at a density of 5×10^5/well onto 96-well plates and allowed to adhere for 24 h. Cells were then exposed for 24 h to glufosfamide. Cells were trypsinized, cell numbers were determined and then the cells were centrifuged at 2500 *g* for 10 min then washed with PBS twice. Cells were then resuspended in ice-cold Cell Lysis Buffer containing 50 mM HEPES, 5 mM DTT, 0.1 mM EDTA, 0.1% CHAPS, at pH 7.4. The cell lysate was centrifuged at 10,000 *g* at 4 °C for 30 min and the cytosolic supernatant was incubated with the fluorogenic caspase-3 tetrapeptide-substrate Ac-DEVD-AMC at a final concentration of 20 μM. Cleavage of the apopain substrate was followed by the determination of emission at 460 nm after excitation at 390 nm using a fluorescence microtiter plate reader.

2.7. Determination of β-glucosidase activity in colon cancer cells

Enzymatic assay of β-glucosidase was achieved according to Chadwick et al. (1995). Briefly, Caco-2, HT29 and T84 cells were cultured for 72 h in 96-well plates as before. Cells were harvested by adding trypsin/EDTA. Cells were then pelleted by centrifugation and washed out twice with PBS. Cell pellets were transferred to cryotubes and then lyophilized in liquid nitrogen. Lyophilized cells were disintegrated using a Vibra-Cell Ultrasonic Dismembrator (Braun Biotech International, Melsungen, Germany). Disintegrated cells were centrifuged in PBS at 10,000 ×*g* for 30 min at 4 °C (Hereaus Instruments, Berlin, Germany). The supernatant was used for the determination of enzymatic activity using *p*-nirophenyl-β-D-glucopyranoside as a substrate. *P*-nitrophenol release was measured colorimetrically at 420 nm for 4 min at 1 min-