



ORIGINAL RESEARCH

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# Dinutuximab beta-targeted therapy kills beta-cell tumors of the pancreas

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## Abstract

**Purpose** Dinutuximab beta is a monoclonal antibody used only in glioblastoma. Streptozotocin is an agent that is particularly toxic to pancreatic beta cells. Dinutuximab beta causes cytotoxicity through natural killer cells and neutrophils and shows effects. In this study, cytotoxicity was induced by streptozotocin without natural killer cells and neutrophils. Gaining the ability to show the effect of Dinutuximab beta without a natural killer was the first aim of this study. This will be especially important in cases where the immune system is deficient, such as cancer. The second aim of the study was to investigate the effects of Dinutuximab beta on cell viability and cell death in insulinoma under the conditions created.

**Methods** The effect of Dinutuximab beta in the presence of natural killer cells in vivo was created by the application of Streptozotocin to Beta-cell tumors of the pancreas in vitro. The cell viability was determined with WST-1 assay. Reactive oxygen species were measured by using dichlorofluorescein diacetate as a spectrophotometer. The cells were marked with DAPI to indicate apoptotic markers (nuclear condensation and fragmentation) with the confocal microscope. *GLUT2* (Glucose transporter 2), *IR* (Insulin receptor), *INS1*, and *INS2* expression levels were analyzed with q-RT-PCR.

**Results** The cell cytotoxicity was induced by Streptozotocin. The cells proliferated with the administration of Dinutuximab beta alone. The result of Dinutuximab beta administered following Streptozotocin administration resulted in more cell death, increased ROS levels, *GLUT2*, *Ins1*, and *Ins2* mRNA expression levels, and decreased *IR* mRNA expression levels. Furthermore, the cells predominantly died via apoptosis showing cytoplasmic condensation and DNA fragmentation.

**Conclusions** The lethal effect of Dinutuximab beta without a natural killer was provided by Streptozotocin in Beta cell tumors of the pancreas.

**Keywords** Insulinoma, INS-1 cell line, Dinutuximab beta, Cell death, Cytotoxicity

## 1 Introduction

Insulinoma is a gastroenteropancreatic neuroendocrine tumor arising from the uncontrolled proliferation of pancreatic  $\beta$  cells. The majority (90%) of insulinomas

are benign. However, it may cause recurrent hypoglycemia as a result of excessive insulin secretion. This makes insulinoma potentially fatal [1, 2]. In addition, incomplete resection after surgery may cause symptoms to persist [2]. The most used insulin-secreting cell lines are beta TC, HIT, RIN, MIN6, and INS-1 cells [3]. INS-1 displays many important characteristics of pancreatic beta cells with its high insulin content in response to glucose [4]. In our study, INS-1 Insulinoma cells were used.

Dinutuximab beta (DB) is a disialoganglioside 2 (GD2) targeted monoclonal antibody that is overexpressed in neuroblastoma cells [5]. It was approved by the European

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Medicines Agency in 2017 for the treatment of high-risk neuroblastoma in patients with minimal response to chemotherapy, stem cell transplantation, age 12 months and older, and relapses [6]. The European Neuroblastoma group of the International Society of Pediatric Oncology currently recommend DB be administered as a continuous intravenous infusion at 10 mg/m<sup>2</sup> daily for ten days as standard care for patients with high-risk neuroblastoma [7].

Immunotherapy has recently resulted in an increase in survival rates for various adult cancers [8]. The increased survival of patients with high-risk neuroblastoma as a result of the application of anti-GD2 therapy in addition to standard care demonstrates the potential and importance of immunotherapy in pediatric oncology [9].

DB-induced cytotoxicity is thought to be mediated mainly by natural killer (NK) cells and neutrophils [10, 11]. Without immunotherapy, T cells cannot recognize neuroblastoma due to low expression of Major Histocompatibility Complexes (MHC-1), and the activity of natural killer (NK) cells is inhibited due to low expression of activating ligands. Immune regulatory mediators in the tumor microenvironment (TME), mesenchymal stem cells (MSC), and myeloid-derived suppressor cells (MDSC) inhibit NK and T cell function. Various targets can be used for immunotherapy [12].

Streptozotocin (STZ) is a toxic glucose analog that can enter pancreatic  $\beta$  cells via glucose transporter 2 (GLUT2). It is widely used to establish experimental diabetes in vivo studies [13]. This model is mainly used to investigate the cytotoxicity of pancreatic  $\beta$  cells after various treatments or to establish hyperglycemia for long-term studies [14, 15]. Oxidative stress and DNA alkylation have been implicated as major mechanisms for STZ-induced pancreatic  $\beta$ -cell injury [16, 17].

In our in vitro study, it was observed that the cells proliferated as a result of DB administration to insulinoma INS-1 cells. Based on the knowledge that this monoclonal immunotherapy drug is effective in cytotoxic conditions, STZ was first applied to the cells. Cell death was performed by applying DB to the cells in which cytotoxicity was created with STZ. This study was an in vitro study so there were no NK and neutrophil cells in the experimental method. Instead of NK and neutrophil cells, we applied STZ to provide the cytotoxic effect of DB. The relationship between STZ and DB is not known in the literature. Therefore, this is the first study in which we showed triggering by STZ of the cytotoxic effect of DB. By determining which type of this cell death is, its effect on insulin secretion and reactive oxygen species at doses causing cell death was investigated. In addition, the protein and mRNA levels of some channel proteins thought to be related to the insulin mechanism were analyzed.

In this context, the effect of DB monoclonal antibody in the treatment of insulinoma was investigated. It has been found that DB, which shows its effect in the presence of NK in vivo, has a similar effect with STZ application in vitro. The known effect of DB for glioblastoma in vivo was demonstrated for the first time in beta tumors, a new type of cancer for this drug. Also, considering that DB is an immune therapy drug, in case of insufficiency of the patient's immune system in cancer, supplementary chemicals may always be needed that can increase the effectiveness of the drug. In this study, a potential chemical that can increase the effectiveness of DB has been demonstrated.

## 2 Material and methods

### 2.1 Materials

HEPES (H3375), trypsin–EDTA (T4049), sodium pyruvate (S8636), 2,7-dichlorofluorescein diacetate (D6883) from Sigma Aldrich (St. Louis, Mo.); RPMI 1640 (21875–034), Penicillin–Streptomycin (10378,016) and Fetal Bovine Serum (26140079) from Gibco (Paisley, Scotland); Bradford Protein Assay (#500–0006), 2-mercaptoethanol (#1610710), iScript™-cDNA-Synthesis kit from Bio-rad (Hercules, California); NucleoSpin® (# 740955.50) RNA II (Macherey–Nagel, Düren, Germany); DNaseI (Macherey–Nagel, Düren, Germany); Applied Biosystems SYBR™ Green PCR supermix (# 4364344) (Applied Biosystems, Waltham, Massachusetts, United States); Phosphatase–Proteinase Inhibitor (A32959) from Thermo Scientific (Rockford, IL); DAPI (28718–90-3) from Merck (Kenilworth, New Jersey), WST1 (11644807001) from Roche was purchased.

### 2.2 Cell culture

The rat insulinoma INS-1 cell line was a generous gift from Prof. Dr. Claes B. Wollheim (University Medical Center, Geneva). INS-1 cells were cultured in 5% CO<sub>2</sub>/95% humidified air at 37°C in RPMI 1640 containing 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin, 10 mM HEPES, 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate. 0.25% trypsin–EDTA was used for the passage of INS-1 cells. After that, the cells were incubated in an incubator with 5% CO<sub>2</sub>/95% humidified air at 37°C for 24 h.

### 2.3 The formation of pancreatic beta-cell cytotoxicity

The pancreatic beta-cell cytotoxicity induced by STZ. STZ dissolved in citrate buffer (CB) (0,1 M, pH 4,6). The different doses of STZ were applied to insulinoma INS-1 cells by WST1 analysis. The IC<sub>50</sub> value was obtained as a result of multiple repetitions. The IC<sub>50</sub> value was found as 3.2373 mM after 2 h of STZ incubation with cells.

## 2.4 Cell viability analysis

$5 \times 10^4$  cells/wells were plated on 96-well plates for WST-1 assay. It was solved STZ in citrate buffer (CB), and DB in the medium (C). Citrate buffer and the medium was given to cells as the control group. The cells were incubated for 2 h for STZ and CB; 24, 48, and 72 h for DB and C; 2 h + 24, 48, and 72 h for STZ + DB and CB + C; 24, 48, and 72 h + 2 h for DB + STZ and C + CB. After that, the medium was removed. 100  $\mu$ l/well fresh medium and 10  $\mu$ l/well WST-1 were added to wells. The cells were incubated in 5% CO<sub>2</sub>/95% humidified air at 37°C for 3 h. After incubation WST-1 results were analyzed by a spectrophotometer. The control value was considered as 100%. The cell viability rate was calculated according to the control.

## 2.5 Cell death detection analysis

$2 \times 10^5$  cells/wells were seeded on coverslips and incubated for 24 h in CO<sub>2</sub> 5% incubators at 37 °C. On the next day, cells were treated with a certain dose of the experimental setup and waited for incubation times. After the incubations, cells were fixed with 4% paraformaldehyde for 15 m at room temperature. After that, the cells were washed with PBS and stored at +4 until studied. It was stained with a mounting medium containing DAPI for condensation and fragmentation. Images were taken using a confocal microscope (Leica, Light Sheed, DMI8).

## 2.6 ROS assay

$5 \times 10^4$  cells/wells were plated on 96-well plates and then the cells were incubated with 5% CO<sub>2</sub> at 37°C. The determined doses were applied to the cells for incubation times. 2,7-dichlorofluorescein diacetate (DCFDA) was used to measure intracellular reactive oxygen species (ROS). INS-1 cells were incubated for half an hour with 10 mM DCFDA. Cells were analyzed on a fluorescent ELISA plate reader. The control group values were accepted as 100%. The values of the experimental group were calculated according to the control group.

## 2.7 mRNA expression analysis

- RNA isolation and cDNA Synthesis

Total RNA was isolated from the cell pellets with NucleoSpin<sup>®</sup> RNA II according to the kit protocol. To digest genomic DNA contamination, RNA samples were treated with DNaseI. RNA concentration and quality were determined with a Nanodrop spectrophotometer. Total RNA samples using 1 mg total RNA were

reverse-transcribed using the iScript<sup>™</sup>-cDNA-Synthesis kit, according to the manufacturer's protocol.

- Real-time PCR

Real-time PCR was performed with Applied Biosystems SYBR<sup>™</sup> Green PCR supermix using Quantstudio 7 real-time PCR device. The primer as follows: Glut2; forward: GCTGGAAGAAGCGTATCAGG, reverse: AATCCTGATTGCCAGAATG; IR; forward: CCTACTGCTATGGGCTCCG, reverse: AGGATCTGCAGATGGCCCTC, Ins-1; forward: GGGGAACGTGGTTTCTTCTAC, reverse: CCAGTTGGTAGAGGGAGCAG, Ins-2; forward: CAGCACCTTTGTGGTTCTCA, reverse: CACTCCAGTGCCAAGGT, Beta Actin; forward: 5'-GGTCGGTGTGAACGGATTGG-3, reverse: 5'-ATGTAGGCCATGAGGTCCACC-3. PCR reactions were set in 12.5  $\mu$ l and melting curve analyses were done. Ct values were converted into arbitrary units using the  $\Delta\Delta$ Ct method. For all the samples primer dimers were checked and confirmed that there were no primer dimers.

## 2.8 Statistical analysis

Multiple comparisons were made with the ANOVA variance analysis test and non-parametric Tukey's test using GraphPad Prism 5 computer software. The results were expressed by  $\pm$  S.E.M. The value of  $p < 0.05$  was considered to be statistically significant. All experiments were repeated in triplicate on different days.

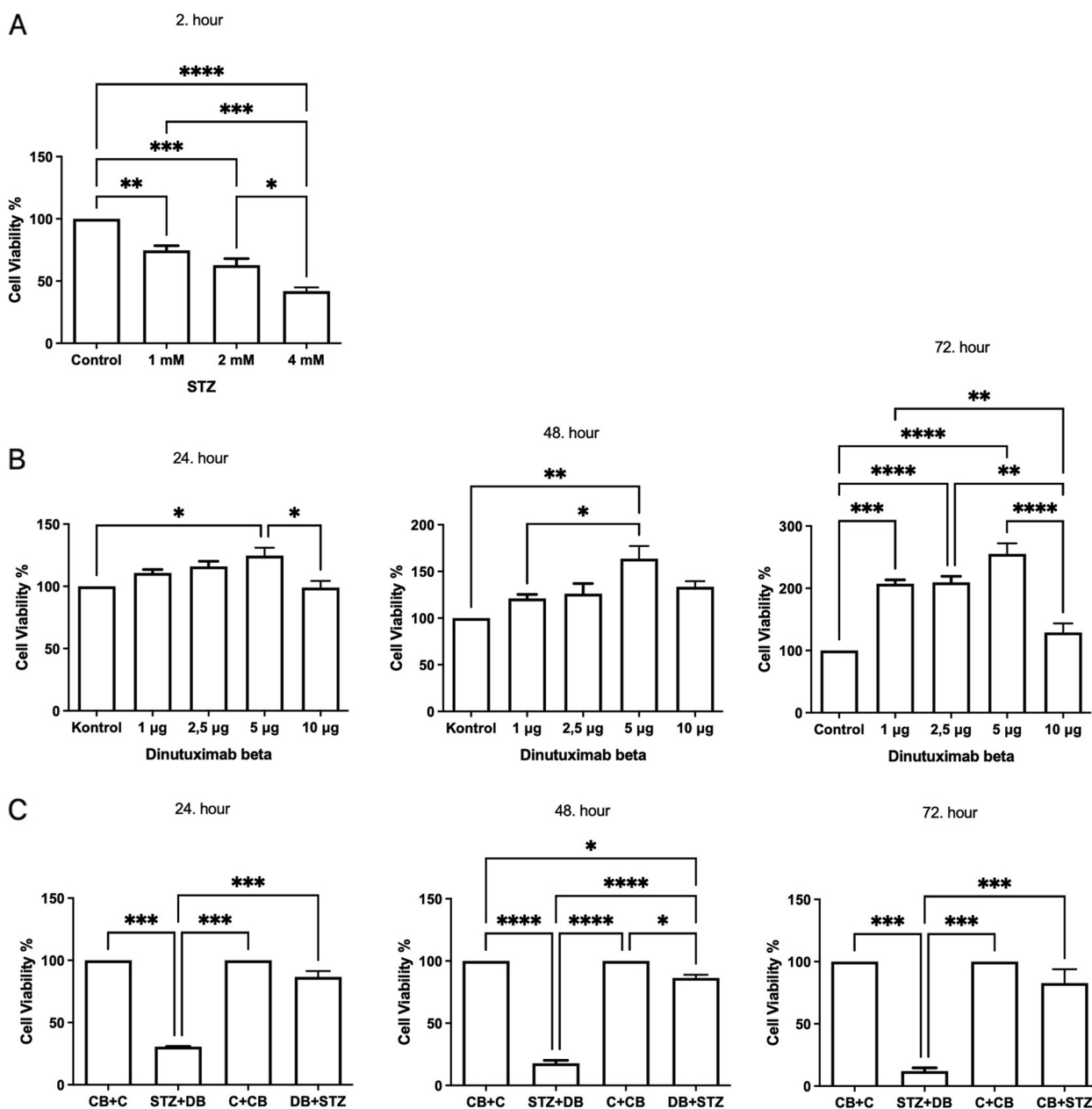
## 3 Results

### 3.1 Dinutuximab beta application to toxicity-induced INS-1 cells reduces cell viability

After calculating the IC<sub>50</sub> value of STZ (Fig. 1A), this dose was applied to INS-1 cells before and after being given DB. Cell proliferation increased over time when DB was administered alone. This proliferation increases in a time-dependent manner (Fig. 1B). Cell viability further decreased over time in the group treated with STZ before DB according to the group given STZ after DB (Fig. 1C). According to these results, the highest proliferation was observed with the administration of only DB, and the maximum decrease in cell viability was observed with the administration of STZ before the DB at the 72nd hour.

### 3.2 Apoptotic cell death occurs with Dinutuximab beta application to toxicity-induced INS-1 cells

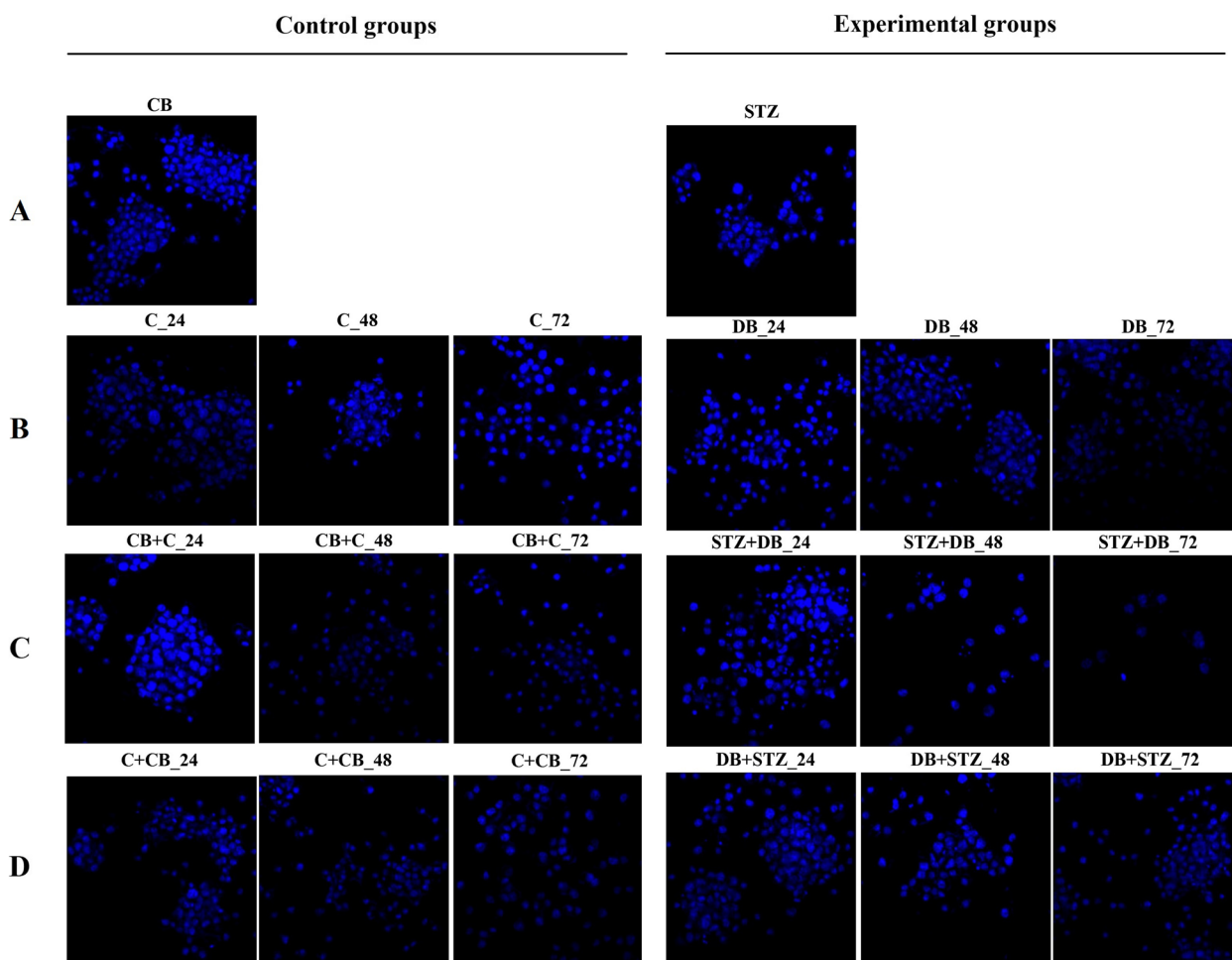
Apoptotic cell death did not occur as a result of the application of STZ to the cells (Fig. 2A). Since cell proliferation occurred as a result of DB application, cell death was not observed (Fig. 2B). DNA condensation and fragmentation caused by apoptosis showed with



**Fig. 1** The cell viability % of insulinoma INS-1 cells with given STZ (A), DB (B), STZ+DB, and DB+STZ (C) and their control groups. For STZ: CB %100 ± 0; 1 mM STZ % 74,59 ± 3,779; 2 mM STZ % 62,76 ± 5,310; 4 mM STZ % 41,96 ± 3,013. For 24h DB: C %100 ± 0; 1 mg DB 110,9 ± 2,678; 2,5 mg DB 115,9 ± 4,087; 5 mg DB 124,7 ± 6,329; 10 mg DB 99,00 ± 5,430; for 48h DB: C %100 ± 0; 1 mg DB 121,2 ± 4,320; 2,5 mg DB 126,3 ± 10,77; 5 mg DB 163,8 ± 13,50; 10 mg DB 133,6 ± 6,067; for 72h DB: C %100 ± 0; 1 mg DB 207,4 ± 6,122; 2,5 mg DB 209,6 ± 9,652; 5 mg DB 255,3 ± 17,12; 10 mg DB 129,0 ± 14,54. For 24h CB+C %100 ± 0; STZ+DB 30,70 ± 0,09765; C+CB %100 ± 0; DB+STZ 86,70 ± 4,718; for 48h CB+C %100 ± 0; STZ+DB 17,84 ± 2,360; C+CB %100 ± 0; DB+STZ 86,40 ± 2,567; for 72h CB+C %100 ± 0; STZ+DB 12,10 ± 2,548; C+CB %100 ± 0; DB+STZ 82,76 ± 11,17

DAPI and this indicates that cells mostly die from apoptosis. Apoptotic cell death occurs in the group treated with DB for 24 and 48 h after STZ application (Fig. 2C). More DNA condensation and fragmentation were observed at 24 h than at 48 h. Although cell death

was much higher at 72 h, this death may not apoptotic cell death due to the absence of DNA condensation and fragmentation. No significant cell death was observed at 24, 48, and 72 h in the STZ-treated group after DB administration (Fig. 2D).



**Fig. 2** DAPI stained image of insulinoma INS-1 cells after treatment with CB and STZ (A), C and DB (B), CB+C and STZ+DB (C), C+CB and DB+STZ (D) groups

**3.3 ROS increases with Dinutuximab beta application to toxicity-induced INS-1 cells**

ROS increased in the STZ-treated group compared to the control group. There was no change in ROS level as a result of DB implementation. ROS increased over time with DB administration after being given STZ compared to control groups. In the group given STZ after DB administration, the level of ROS increased as a result of long-term incubation (Fig. 3).

**3.4 *INS1*, *INS2*, and *GLUT2* mRNA expression increases while *IR* mRNA expression decreases with Dinutuximab beta application to toxicity-induced INS-1 cells**

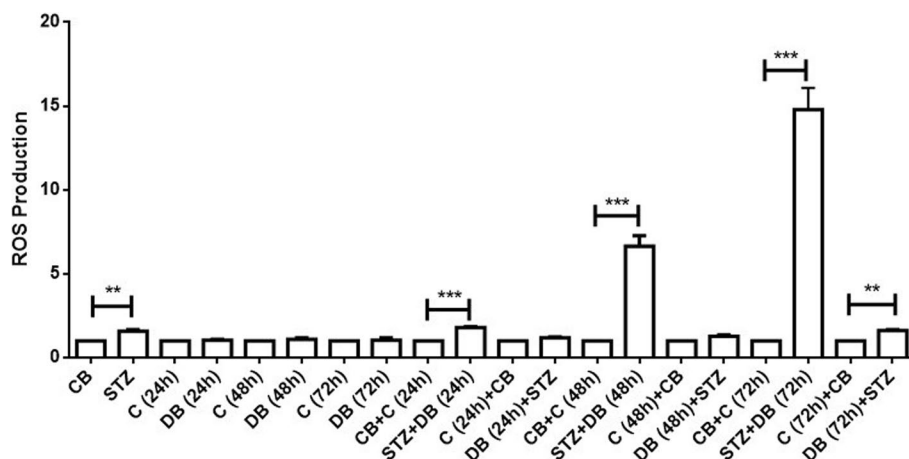
*INS1* and *INS2* mRNA expression levels increased in the STZ-treated group compared to the control group. *INS1* and *INS2* mRNA expression levels decreased with DB administration after given STZ according to given STZ after DB administration. While this expression level

increased in given DB after STZ at 48 h, it was further decreased in given STZ after DB administration. At 72 h, *INS1* and *INS2* mRNA expression levels increased with DB administration after being given STZ. In the group given STZ after DB administration, *INS1* and *INS2* mRNA expression levels increased at 72 h compared to 48 h but decreased compared to the control group (Fig. 4A-B).

*IR* mRNA expression levels decreased at 24 and 72 h with DB application. Expression levels of this mRNA decreased in the DB treatment group after STZ administration at 24, 48, and 72 h. A decrease was observed only in the 48th hour in the group in which STZ was administered after DB administration (Fig. 4C).

*GLUT2* mRNA expression level decreased with STZ application. An increase was observed at 48. hours of DB administration in *GLUT2* mRNA expression levels. *GLUT2* mRNA expression decreased with given DB after STZ administration at the 24th hour, while the





**Fig. 3** ROS production of insulinoma INS-1 cells after treatment with STZ, DB, STZ+DB, DB+STZ and their control groups. For STZ: CB  $1,000 \pm 5,774e-007$ ; STZ  $1,577 \pm 0,09305$ ; C (24h)  $1,000 \pm 5,774e-007$ ; DB (24h)  $1,030 \pm 0,06473$ ; C (48h)  $1,000 \pm 5,774e-007$ ; DB (48h)  $1,091 \pm 0,08671$ ; C (72h)  $1,000 \pm 5,774e-007$ ; DB (72h)  $1,047 \pm 0,1235$ ; CB+C (24h)  $1,000 \pm 5,774e-007$ ; STZ+DB (24h)  $1,791 \pm 0,05649$ ; C (24h)+CB  $1,000 \pm 5,774e-007$ ; DB (24h)+STZ  $1,172 \pm 0,06823$ ; CB+C (48h)  $1,000 \pm 5,774e-007$ ; STZ+DB (48h)  $6,654 \pm 0,5947$ ; C (48h)+CB  $1,000 \pm 5,774e-007$ ; DB (48h)+STZ  $1,278 \pm 0,1308$ ; CB+C (72h)  $1,000 \pm 5,774e-007$ ; STZ+DB (72h)  $14,79 \pm 1,290$ ; C (72h)+CB  $1,000 \pm 5,774e-007$ ; DB (72h)+STZ  $1,615 \pm 0,09275$

expression of this mRNA increased at the 48th and 72nd hours. In the group given STZ after DB administration, *GLUT2* mRNA expression increased at the 24th hour and decreased at the 48th hour (Fig. 4D).

#### 4 Discussion

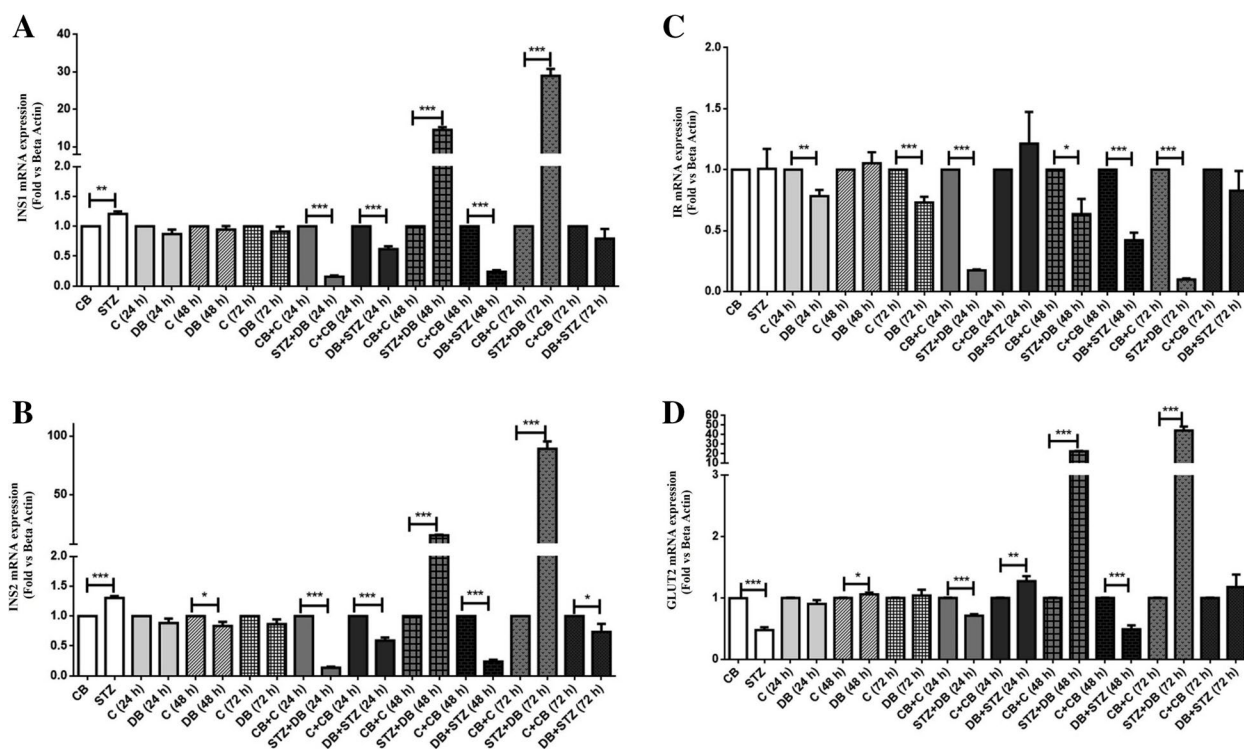
Treatment with antibodies targeting the disialoganglioside GD2 antigen, which is found at high levels in neuroblastoma patients, increased the survival rates of patients with high-risk neuroblastoma [18–20]. DB is an antibody that also binds to GD2. Its effect on pancreatic tumors is unknown.

The anti-GD2 antibody, DB, recruits neutrophils and natural killer (NK) cells to mediate antibody-dependent cellular cytotoxicity (ADCC) [10, 21, 22]. In other words, the anti-GD2 antibody shows its effect in relation to cell cytotoxicity. Based on this situation, cell toxicity was created in insulinoma INS-1 cells, which is a pancreatic tumor, in the first place. Then, DB was given to cells with cytotoxicity. In another group, DB was given first and then toxicity was created with STZ. In this way, the effect of DB on pancreatic tumors was investigated.

Immunotherapeutic treatments for pancreatic cancer have not been successful with very few exceptions [23]. This is most likely due to the late diagnosis of pancreatic cancer [24]. In a study, it was shown that Gemcitabine has immunomodulatory properties in pancreatic cancer [25]. Research has been conducted on developing new chemotherapeutic combinations for pancreatic cancer. Chemotherapy agents such as pomalidomide, oxaliplatin, and zoledronic acid were combined with Gemcitabine

and applied to pancreatic cancer cell lines. As a result, combined immunogenic chemotherapy was provided to be more effective [26]. However, the effects of DB on pancreatic cancer are unknown. In this study, conducted for the first time by us, it was shown that when DB is applied to Beta-cell tumors of the pancreas, it causes the cells to proliferate. However, if DB is administered after toxicity has been induced with STZ, the cells die depending on time. Conversely, when toxicity is induced with STZ after applying DB to the cells, the cells do not die in the same way. This confirmed that previously known DB acts in cytotoxic conditions and showed that it has important survival effects on pancreatic cancer. While DB causes significant death in toxic conditions, in the case of STZ given after DB, the continuation of cell viability occurred, which is thought to be caused by different mechanisms. In other words, the difference between the proteins which active or inactive in DB after toxicity occurs with STZ, and the proteins that are active or inactive in given DB before toxicity occurs later may be the reason for this situation that different cell viability rates.

Cancer cells die with different types of death, such as necrosis, apoptosis, necroptosis, pyroptosis, or ferroptosis, depending on the stimulation or inhibition of anti-cancer immunity [27–29]. Apoptosis is crucial for regulating cell populations during tissue development and the cell cycle. In addition, cytotoxic T lymphocytes induce cell death in target cells or infected cells [30]. Anti-GD2 monoclonal antibodies target GD2-expressing tumor cells. It causes phagocytosis with antibody-dependent cell-mediated cytotoxicity.



**Fig. 4** INS1 (A), INS2 (B), IR (C) and GLUT2 (D) mRNA expression levels of insulinoma INS-1 cells after treatment with STZ, DB, STZ+DB, DB+STZ and their control groups. For INS1: CB  $1,000 \pm 2,500e-007$ ; STZ  $1,207 \pm 0,04333$ ; C (24 h)  $1,000 \pm 2,500e-007$ ; DB (24h)  $0,8725 \pm 0,07296$ ; C (48h)  $1,000 \pm 2,500e-007$ ; DB (48h)  $0,9450 \pm 0,05979$ ; C (72h)  $1,000 \pm 2,500e-007$ ; DB (72h)  $0,9125 \pm 0,08509$ ; CB+C (24h)  $1,000 \pm 2,500e-007$ ; STZ+DB (24h)  $0,1575 \pm 0,01931$ ; C (24h)+CB  $1,000 \pm 2,500e-007$ ; DB (24h)+STZ  $0,6175 \pm 0,05121$ ; CB+C (48h)  $1,000 \pm 2,500e-007$ ; STZ+DB (48h)  $14,47 \pm 0,7534$ ; C (48h)+CB  $1,000 \pm 2,500e-007$ ; DB (48h)+STZ  $0,2400 \pm 0,02858$ ; CB+C (72h)  $1,000 \pm 2,500e-007$ ; STZ+DB (72h)  $28,92 \pm 1,871$ ; C (72h)+CB  $1,000 \pm 2,500e-007$ ; DB (72h)+STZ  $0,7975 \pm 0,1606$ . For INS2: CB  $1,000 \pm 2,500e-007$ ; STZ  $1,307; 0,02963$ ; C (24 h)  $1,000 \pm 2,500e-007$ ; DB (24h)  $0,8875 \pm 0,07510$ ; C (48h)  $1,000 \pm 2,500e-007$ ; DB (48h)  $0,8375 \pm 0,06909$ ; C (72h)  $1,000 \pm 2,500e-007$ ; DB (72h)  $0,8675 \pm 0,07857$ ; CB+C (24h)  $1,000 \pm 2,500e-007$ ; STZ+DB (24h)  $0,1375 \pm 0,01652$ ; C (24h)+CB  $1,000 \pm 2,500e-007$ ; DB (24h)+STZ  $0,5900 \pm 0,05307$ ; CB+C (48h)  $1,000 \pm 2,500e-007$ ; STZ+DB (48h)  $15,42 \pm 0,4965$ ; C (48h)+CB  $1,000 \pm 2,500e-007$ ; DB (48h)+STZ  $0,2425 \pm 0,02869$ ; CB+C (72h)  $1,000 \pm 2,500e-007$ ; STZ+DB (72h)  $89,11 \pm 6,170$ ; C (72h)+CB  $1,000 \pm 2,500e-007$ ; DB (72h)+STZ  $0,7375 \pm 0,1352$ . For IR: CB  $1,000 \pm 2,500e-007$ ; STZ  $1,008 \pm 0,1629$ ; C (24 h)  $1,000 \pm 2,500e-007$ ; DB (24h)  $0,7825 \pm 0,04973$ ; C (48h)  $1,000 \pm 2,500e-007$ ; DB (48h)  $1,053 \pm 0,09003$ ; C (72h)  $1,000 \pm 2,500e-007$ ; DB (72h)  $0,7300 \pm 0,04813$ ; CB+C (24h)  $1,000 \pm 2,500e-007$ ; STZ+DB (24h)  $0,1725 \pm 0,008539$ ; C (24h)+CB  $1,000 \pm 2,500e-007$ ; DB (24h)+STZ  $1,213 \pm 0,2596$ ; CB+C (48h)  $1,000 \pm 2,500e-007$ ; STZ+DB (48h)  $0,6350 \pm 0,1241$ ; C (48h)+CB  $1,000 \pm 2,500e-007$ ; DB (48h)+STZ  $0,4200 \pm 0,06403$ ; CB+C (72h)  $1,000 \pm 2,500e-007$ ; STZ+DB (72h)  $0,09750 \pm 0,01109$ ; C (72h)+CB  $1,000 \pm 2,500e-007$ ; DB (72h)+STZ  $0,8275 \pm 0,1606$ . For GLUT2: CB  $1,000 \pm 2,500e-007$ ; STZ  $0,4775 \pm 0,04661$ ; C (24 h)  $1,000 \pm 2,500e-007$ ; DB (24h)  $1,000 \pm 2,500e-007$ ; DB (48h)  $1,060 \pm 0,02828$ ; C (72h)  $1,000 \pm 2,500e-007$ ; DB (72h)  $1,040 \pm 0,09695$ ; CB+C (24h)  $1,000 \pm 2,500e-007$ ; STZ+DB (24h)  $0,7125 \pm 0,02689$ ; C (24h)+CB  $1,000 \pm 2,500e-007$ ; DB (24h)+STZ  $1,275 \pm 0,08088$ ; CB+C (48h)  $1,000 \pm 2,500e-007$ ; STZ+DB (48h)  $22,54 \pm 0,2165$ ; C (48h)+CB  $1,000 \pm 2,500e-007$ ; DB (48h)+STZ  $0,4900 \pm 0,06468$ ; CB+C (72h)  $1,000 \pm 2,500e-007$ ; STZ+DB (72h)  $44,22 \pm 4,286$ ; C (72h)+CB  $1,000 \pm 2,500e-007$ ; DB (72h)+STZ  $1,180 \pm 0,2000$

Complement-dependent cytotoxicity also causes lysis of the cell. It induces cell death, which leads to apoptosis and necrosis [31]. Anti-GD2 mAbs can induce apoptotic and necrotic cell death in GD2-positive tumor cells without the involvement of immune mechanisms [32, 33]. In this study, cell death occurred depending on time as a result of DB applied after cytotoxicity of the Beta-cell tumors of the pancreas. While this cell death was more apoptotic cell death at 24 h, cell death increased at 48 h and apoptotic cell death decreased. At 72 h, while the maximum cell death was observed, apoptotic cell death was not observed. Cell death was not observed when cytotoxicity was induced in cells after DB application.

ROS is formed as a product of oxygen metabolism. Cell signaling and homeostasis also play an important role [34]. Certain levels of ROS promote cell proliferation and cell survival, while too high levels damage cells and cell components. In this way, lipid peroxidation and DNA damage increase, and the cell dies by apoptosis [35]. According to our results, ROS levels increased as cell damage increased. In the results, ROS and apoptotic cell death did not progress in parallel. The ROS level is highest in cases where apoptotic cell death is not observed but cell viability decreases the most. In this case, we can say that the change in the cells after cytotoxicity interacts with the applied DB, on the contrary when DB is applied

first and then cytotoxicity is created, there is no such effect.

In our previous study, we observed that *INS1* and *INS2* mRNA expressions decreased, *IR* mRNA expression did not change, and *GLUT2* mRNA expression increased in the group in which apoptotic cell death occurred as a result of exogenously administered substances in insulinoma cells [36]. In this study, similar to the other study, we observed that *INS1* and *INS2* mRNA expressions were decreased in the group with apoptotic cell death in which DB was applied for 24 h after cytotoxicity. However, we see that *INS1* and *INS2* mRNA expressions increase with increased cell death at 48 and 72 h, but decreased apoptosis may indicate that there should be another cell death mechanism. Therefore, since cell damage does not occur in apoptotic cell death, the expression of related genes decreases with decreasing cell viability. However, in cases where there is cell damage, it can be thought that it is desirable to increase insulin production, which is very important and typical especially for pancreatic beta cells, in order to protect the remaining cells [37]. Likewise, since there was no cell damage in the group in which cellular cytotoxicity was formed after DB administration, the expression of the relevant genes decreased due to the decreased cell viability. The level of *IR* mRNA expression was also decreased in both groups in which DB was given after and before, depending on the death of the cells and the decrease in their viability. Glucose will be taken into the cell via *GLUT2*. According to the results of the study, increased *GLUT2* mRNA expression may have caused excessive glucose entry into the cell. This means more insulin secretion from the cell. Therefore, the change in *INS1* and *INS2* mRNA expressions and the change in *GLUT2* mRNA expression is in the same direction.

## 5 Conclusions

In our study, DB showed an effect in the presence of cytotoxicity induced by STZ without NK cells for the first time. It is a very promising finding that this drug can be effective without immune cells, due to the low immune system in diseases such as cancer. This study, which was performed for the first time showed that DB can be effective in pancreatic tumors. In addition, it is necessary to test its usability for the treatment of pancreatic cancer with in vivo experiments.

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### Authors' contributions

Ayse Karatug Kacar<sup>1</sup>, created the main idea, experimental design, all cell culture experiments done, determination of cell viability, ROS levels, and mRNA

expression by qRT-PCR, evaluation of all experimental results, done statistics, and written manuscript. Zelal Adiguzel<sup>2</sup>, determination of cell death detection, ROS levels, and mRNA expression by qRT-PCR, edited manuscript. The author(s) read and approved the final manuscript.

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### Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### Declarations

#### Competing interests

The authors declare no conflict of interest.

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