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ORIGINAL ARTICLE

Anti-tumor properties of the cGMP/protein kinase G inhibitor DT3 in pancreatic adenocarcinoma

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Abstract Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers in the world. Therefore, new therapeutic options are urgently needed to improve the survival of PDAC patients. Protein kinase G (PKG) conducts the interlude of cGMP signaling which is important for healthy as well as for cancer cells. DT3 is a specific inhibitor of PKG, and it has been shown to possess an anti-tumor cytotoxic activity in vitro. The main aim of this work was to investigate anti-tumor effects of DT3 upon PDAC in vivo.

Expression of PKG was assessed with real-time PCR analysis in the normal and tumor pancreatic cells. In vitro cell viability, proliferation, apoptosis, necrosis, migration, and invasion of the murine PDAC cell line Panc02 were assessed after DT3 treatment. In vivo anti-tumor effects of DT3 were investigated in the murine Panc02 orthotopic model of PDAC. Western blot analysis was used to determine the phosphorylation state of the proteins of interest.

Functional PKGI is preferentially expressed in PDAC cells. DT3 was capable to reduce viability, proliferation, and migration of murine PDAC cells in vitro. At the same time, DT3 treatment did not change the viability of normal epithelial cells of murine liver. In vivo, DT3 treatment reduced the tumor volume and metastases in PDAC-bearing mice, but it was ineffective to prolong the survival of the tumor-bearing animals. In addition, DT3 treatment decreased phosphorylation of GSK-3, P38, and CREB in murine PDAC.

Inhibition of PKG could be a potential therapeutic strategy for PDAC treatment which should be carefully validated in future pre-clinical studies.

Keywords Pancreatic adenocarcinoma · DT3 · Protein kinase G · Panc02 · Orthotopic mouse model

Abbreviations

GC	Guanylyl cyclase
PDAC	Pancreatic ductal adenocarcinoma
PKG	Protein kinase G

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers in the world. PDAC mortality increases modestly in countries of southern and central/eastern Europe (Bosetti et al. 2012). Patients with PDAC have a very poor prognosis with a five-year survival rate of <1 % and a median survival of 6 months (Siegel et al. 2012). Upon tumor resection, a 5-year survival rate increases to approximately 15 %, while a 25 % survival is attained in the context of adjuvant chemotherapy (Warshaw and Fernandez-del Castillo 1992). The reasons for this poor prognosis are PDAC early dissemination, lack of early specific symptoms, and late diagnosis (Rosty and Goggins 2002). Additionally, PDAC is highly resistant to chemotherapy and targeted therapy, and withstand immunotherapy (Bazhin et al. 2014). Currently, regimens based on

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gemcitabine or 5-fluorouracil constitutes the standard therapeutic approach to advanced PDAC. In the palliative situation, recently published data from the FOLFIRINOX trial revealed a significant survival advantage, but this was associated with an increased toxicity (Conroy et al. 2011). Moreover, the overall clinical impact of these conventional treatment options is rather disappointing. Therefore, new therapeutic options are urgently needed to improve the survival of PDAC patients.

Protein kinase G (PKG) conducts the interlude of cGMP signaling. PKG is commonly activated when cGMP signaling is mediated by NO (Francis et al. 2010). NO at a nanomolar concentration binds to haem, a prosthetic group of soluble guanylyl cyclase (GC), and causes a 100- to 200-fold activation of the enzyme (Cary et al. 2006). Activation of NO-GC increases conversion of GTP to cGMP, resulting in the elevation of cGMP, which initiates the cGMP signaling pathway and subsequent physiological changes (Bryan et al. 2009).

cGMP/PKG signaling is also important for cancer cells. It has been demonstrated that the basal activity of PKG is essential for preventing spontaneous apoptosis of cancer cells (Fraser et al. 2006). Recently, our group has found out that the PKG inhibitor DT3 can reduce the viability and proliferation as well as migration of human PDAC cells in vitro (Karakhanova et al. 2014a), pointing to the therapeutic potential of PKG inhibition for PDAC. DT3 is a specific membrane-permeant peptide-based inhibitor of PKG. Biochemically, this substance inhibits PKG at a site different from the catalytic domain, making DT3 a competitive/noncompetitive inhibitor at a nanomolar concentration (Dostmann et al. 2000). Influence of DT3 on PDAC cells in vivo is so far unclear. There is evidence showing that the DT3 homolog—DT2 is a specific PKGI inhibitor only in vitro but not in vivo (Gambaryan et al. 2012), whereas the data upon the ability of DT3 to inhibit PKGI in vivo are absent.

The main aim of this work was to investigate DT3 effects on tumor cells in vivo using a murine PDAC model. We showed that DT3 was capable to reduce the viability, proliferation, and migration of the murine PDAC cells in vitro. In vivo, DT3 treatment reduced the tumor volume and metastases in PDAC-bearing mice. However, the treatment was ineffective regarding survival of the tumor-bearing animals.

Materials and methods

Materials

The membrane-permeant peptide-based inhibitor of PKGIα—DT3—was purchased from BIOLOG Life Science Institute (Germany). A goat polyclonal (monospecific) antibody against PKGIα/β and secondary anti-rabbit antibody were purchased from Santa Cruz Biotechnology, Inc. (USA). Antibodies against phosphorylated and unphosphorylated forms of CREB, ERK1/2, GSK−3, JNK, and P38 were purchased from Cell Signaling Technology, Inc. (USA).

Cell lines

The murine pancreatic adenocarcinoma cell line Panc02 cell line was originally from (Corbett et al. 1984). Cells were cultivated in RPMI-1640 medium with 10 % fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, PAA Laboratories (Germany). The murine pancreatic adenocarcinoma cell line LTPA was purchased by ATCC (ATCC® CRL-2389™, VA USA). The LTPA cells were cultivated in the Eagle's Minimum Essential Medium supplemented with 10 % fetal bovine serum. The epithelial liver cell line NCTC clone 1469 was purchased from CLS GmbH (Germany). The cells were cultivated in DMEM supplemented with 4 mM L-glutamine, 0.15 % sodium bicarbonate, 0.45 % glucose, and 10 % horse serum. All cells were cultivated at 37 °C and 5 % CO_2 and routinely checked for mycoplasma contamination.

Panc02 murine tumor model

Syngeneic pancreatic carcinoma cells (Panc02) were injected orthotopically in C57Bl/6 mice as described elsewhere (Shevchenko et al. 2013). Mice were narcotized with isofluran/O2 inhalation. After achieving surgical tolerance (stage III2), mice were opened with an abdominal section. Five microliters of Panc02 cells in a concentration of 2×10^7cells/ml in PBS were injected in the pancreatic head with a 25-μl gastight syringe (Hamilton, Reno, USA). The injection site was clamped for about 30 s after removal of the syringe. The tissue was carefully returned to its original position, and all layers of the wound were separately sutured with synthetic absorbable sutures material (polysorb 6–0, Tyco Healthcare). For the therapeutic experiments, mice were treated i.p. with DT3 (5 mg/kg/day in isotonic NaCl solution) on days 5, 7, and 9. Control treatment was done with vehicle (isotonic NaCl solution). Tumor volume was measured at day 21 after cell transplantation using a vernier caliper and applying the following formula: (length × height × width) / 6. Animal experiments were carried out under the pathogen-free conditions in the animal facility of the University Heidelberg after approval by the authorities (Regierungspraesidium Karlsruhe).

RNA isolation, conventional, and real-time RT-PCR analyses

Total RNA from cell lines was isolated using a RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. RNA concentrations were determined using a NanoPhotometer™ Pearl (SERVA Electrophoresis, Germany). RT-PCR primers for murine PKGI, PKGII, β-

actin, and GAPDH were purchased from Qiagen (Germany). Conventional RT-PCR analysis of PKGI and PKGII was performed as described elsewhere (Bazhin et al. 2008). Briefly, 1 μg of the total RNA from cell lines or tissues was reverse-transcribed by using the 1st strand complementary DNA (cDNA) synthesis kit (Roche Diagnostics, Germany) at 42 °C for 50 min as described by the manufacturer. PCR amplification was performed using 1 μl from the RT reaction mixture in 25 μl of the PCR mixture containing 50 pmol of sense and antisense primers. After the initial incubation at 94 °C for 2 min, 33 cycles of amplification were carried out for PKGI and PKGII, and 21 cycles for GAPDH. At least three independent PCR experiments were running.

Real-time RT-PCR analysis was done as described elsewhere (Zhu et al., 2014), using the SYBR-Greensystem, and measured using a Light-Cycler (Roche,Germany). For each experiment, a melting curve analysis and a gel electrophoresis of PCR products were performed to exclude primer dimers. The data were analyzed using the comparative Ct method (Schmittgen and Livak 2008). Each measurement was performed in a technical duplicate.

Western blot analysis

Western blot analysis was performed as described elsewhere (Karakhanova et al. 2014b). Briefly, 10 μg of total protein per lane were separated in a 12.5 % polyacrylamide gel. After SDS-PAGE, separated proteins were electrotransferred from the gel to PVDF membranes (Immobilon; Millipor) in a Tris-glycine-methanol buffer (pH 8.3). After blocking with 5 % (*w*/*v*) of BSA in PBS, containing TWEEN-20, the membranes were incubated overnight with an antibody against the protein of the interest. All antibodies were diluted as suggested by the manufacturer. Then, membranes were washed and incubated for 1.5 h with an HRP-conjugated secondary IgG by 1:10,000 dilutions. Immunoreactive bands were visualized and analyzed by an enhanced chemiluminescence (ECL) system (Amersham Bioscience) using Transilluminator (Peqlab) and ImageJ software according to the method described by the manufacturer.

Measurement of PKG activity

Measurement of the PKG activity in lysates of PDAC cells was done using the CycLex Cyclic GMP-dependent protein kinase (cGK) Assay Kit (CycLex Co., Ltd., Japan) as described previously (Karakhanova et al. 2014). Briefly, the cell pellet (5–10 × 10^6 cells) was resuspended in PBS with a proteinase inhibitor cocktail. The cells were lysed by three freeze-thaw cycles followed by centrifugation at 400*g* for 10 min at 4 °C. One hundred microliters of supernatant (10 mg/ml of total proteins) were incubated for 10 min with DT3 (solved in PBS) at concentrations mentioned in the figure legends or with PBS alone as a vehicle control (co). Then, 10 μL of supernatant were added into the wells pre-coated with a substrate corresponding to the recombinant PKG substrate. Afterwards, 90 μL of the cGMP plus Kinase Reaction Buffer from the kit were added, and the wells were incubated for 30 min at 30 °C. After washing of the wells, 100 μL of the HRP conjugated Detection Antibody 10H11 were added into each well, and the plate was incubated at room temperature for 60 min. After a washing step, 100 μL of the substrate reagent were pipetted and incubated at room temperature for 15 min. Finally, 100 μL of the Stop Solution was added into each wells, and the absorbance was measured using a plate reader at 450 nm. The cGK positive control-1 (Catalytic domain) obtained from CycLex Co., Ltd. (Japan) was used as a positive control. As a negative control, a probe without adding of the protein supernatant was used. The PKG activity expressed in optical density (OD) was measured in duplicate, and the final activity was calculated from three independent experiments.

Cell viability and proliferation assays

Cell viability after DT3 treatment was analyzed both with a EZ4U Kit (Biomedica, Austria) and with a ApoTox-Glo™ Triplex Assay Kit (Promega, USA), and proliferation was assessed with a BrdU Cell Proliferation Assay Kit (Millipore, USA), as previously described (Karakhanova et al. 2014b). At least three independent experiments were performed for each assay.

Apoptosis and necrosis assays

Apoptosis after DT3 treatment was measured using the ApoTox-Glo™ Triplex Assay Kit (Promega, USA) and the Annexin V and ethidium homodimer III staining with the Apoptotic/Necrotic Cells Detection Kit (PromoKine, Germany) as described previously (Karakhanova et al. 2014b). Ten micromolar staurosporine or 100 μM ionomycin were used as a positive control for apoptosis or necrosis, respectively. The apoptosis and necrosis tests were performed for at least three independent experiments.

Migration and invasion assays

Migration and invasion assays were performed with a CytoSelect™ Cell Migration and Invasion Assay Kit (Cell Biolabs, Inc., USA), as described previously (Karakhanova et al. 2014b) for three independent experiments.

Statistical analysis

All statistical analyses were done using GraphPad Prism Version 5.01. For in vitro experiments, distributions of continuous variables were described by their means and SE, and

were presented as column bar graphs. The null hypothesis (mean values were equal) versus the alternative hypothesis (mean values were not equal) was tested by unpaired two-tailed *t* test. In in vivo experiments, the continuous variables (tumor growth in mm^3) were normally distributed and were presented as a vertical scatter plot with mean and SE. The null hypothesis (mean values were equal) versus the alternative hypothesis (mean values were not equal) was tested by the unpaired two-tailed *t* test. Survival probabilities were estimated using the Kaplan-Meier method. The null hypothesis (survival curves were equal) versus the alternative hypothesis (survival curves were not equal) was tested by the log-rank (Mantel-Cox) test. Qualitative data (number of mice with metastases) were analyzed with χ^2 method. A p value <0.05 was considered significant.

Results

Preferential expression of PKG and the PKG activity in murine PDAC cells

First, we analyzed expression of PKGI and PKGII in pancreatic tissues from healthy mice and PDAC tissues from tumor-bearing mice using real-time RT-PCR analysis. In the cell culture model, we employed the murine PDAC cell line Panc02; NCTC cells of normal murine liver epithelium was used as control cells because of the absence of an established cell line of healthy murine pancreas. The analysis revealed a higher PKGI expression and a lower PKGII expression in the murine PDAC cells in comparison with the control cells (Fig. 1a). It should be noted that in tumor cells, PKGII was generally lower expressed than PKGI, whereas in healthy cells, the reversed picture likely took place. Panc02 cells showed the PKG kinase activity, and the PKGI specific inhibitor DT3 at 1-μM concentration significantly reduced this activity (Fig. 1b). Thus, we can suppose that functional PKGI is expressed in murine PDAC cells.

DT3 induces cytotoxicity through necrosis and reduces proliferation and migration but not invasion of PDAC cells

Next, we wanted to answer the question as to whether DT3 possesses cytotoxicity in respect of the murine PDAC cell line Panc02 which is used for the orthotopic transplantation in BL6 mice. As expected, DT3 at 1-μM concentration induced cytotoxicity of PDAC cells measured both with the ApoTox-Glo™ Triplex Assay Kit (Fig. 2a) and with a conventional MTT test (data not shown). The IC50 for the DT3 cytotoxicity was calculated as to be 0.9 μM (data not shown). The DT3-induced cell death mechanism was mainly necrosis and late apoptosis (Fig. 2e). DT3 also reduced the proliferation and migration of the Panc02 cells, but did not affect their invasion (Fig. 2b, c, d). Cytotoxicity of DT3 was confirmed with the additional mutine PDAC cell line LPTA (Fig. 2e). In contrast, treatment of the normal murine liver epithelium NCTC cells with DT3 did not obviously induce their cytotoxicity (Fig. 2g). Based on these data, we decided to assess the anti-cancer properties of DT3 in vivo.

DT3 treatment of PDAC-bearing mice reduces the PDAC tumor volume and a number of colon metastases but does not affect survival of the mice

Since this work was only a "prove of principle" that the pharmacological inhibition of PKG would have certain anti-cancer properties, we did not perform a detailed toxicological investigation of DT3 in vivo. Instead of this, we decided to treat the PDAC-bearing mice with DT3 i.p. (5 mg/kg/day) on days 5, 7, and 9. This dose was tolerated well; solely, the motor activity of the treated mice was reversibly reduced during about 5 min after drug injection.

We found out that DT3 treatment reduced the tumor volume of PDAC-bearing mice (Fig. 3a). Furthermore, this treatment diminished a number of mice with colon metastases, and had a tendency to reduce liver metastases (Fig. 3b). However, despite the effect on the tumor volume, DT3 did not prolong survival of the PDAC-bearing mice ($p = 0.39$, Fig. 3c), showing only marginal difference in the median survival (29.5 days for the control-treated mice median survival and 33.0 days for the DT3-treated animals).

DT3 decreases phosphorylation of GSK−3, P38, and CREB in murine PDAC tumors

Earlier, we have showed that in human PDAC cells, the phosphorylation of CREB, ERK1/2, GSK−3, JNK1, JNK2/3, and P38 can be affected by DT3 (Karakhanova et al. 2014b). Now, we have analyzed the impact of DT3 on in vivo phosphorylation of these signaling proteins in tumors of PDAC-bearing mice after their treatment with DT3 (Fig. 4). For this purpose, the Panc02 cells were treated 24 h with 0.9 μM DT3. Afterwards, the live cells were analyzed with Western blot as described in "Materials and methods". It turned out that DT3 decreased the phosphorylation of P38 significantly and showed a good tendency toward the inhibition of phosphorylation of GSK−3 and CREB (Fig. 4). Whereas no DT3 effects on phosphorylation of ERK1/2, JNK1 and JNK2/3 were registered in the experimental conditions used.

Discussion

As mentioned in "Introduction," until now, there is no possibility to cure patients with PDAC at the moment of diagnosis.

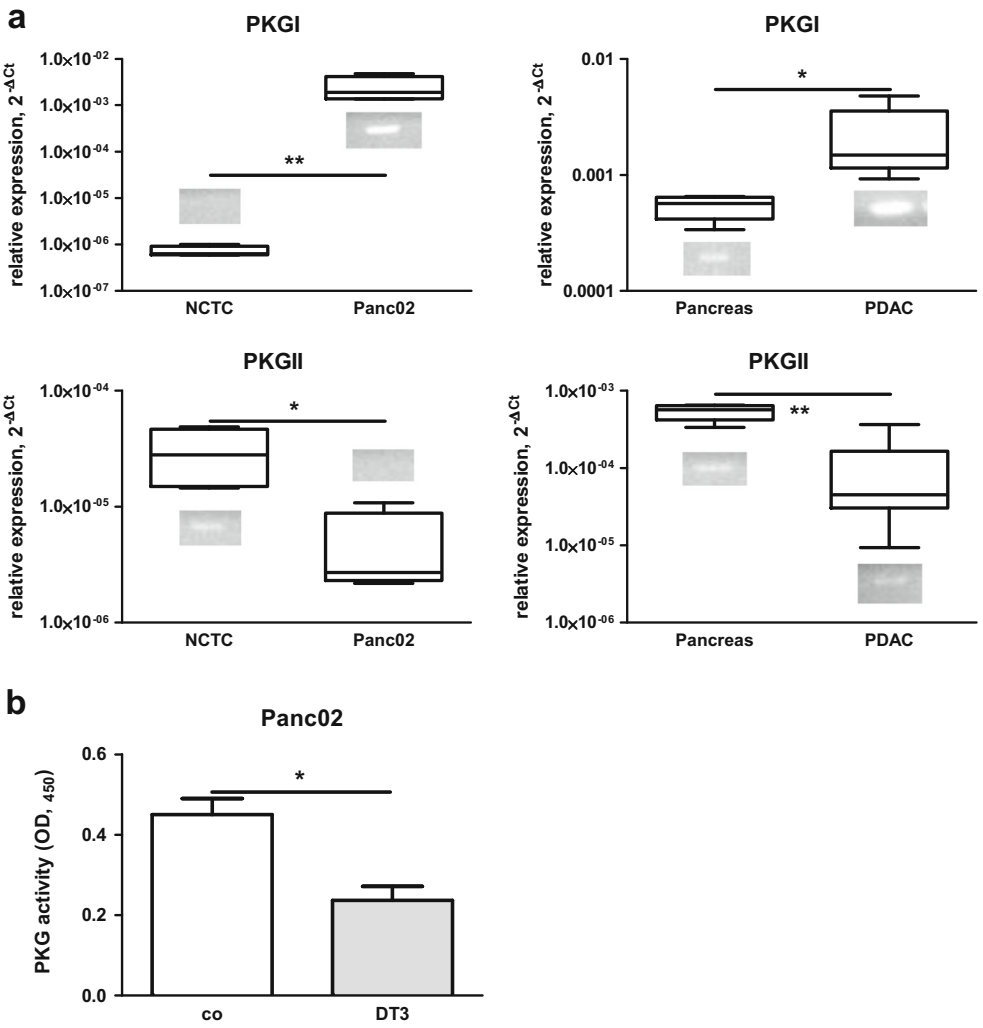

Fig. 1. Preferential expression of PKG and PKG activity in murine PDAC cells. **a** Real-time PCR analysis of PKGI and PKGII expression in the cells of normal liver epithelial (NCTC) and of the murine PDAC cell line Panc02 (*left panel*), or in the tissue of normal murine pancreas and of murine PDAC (*right panel*). The embedded pictures present the agarose gel electrophoresis of PCR products. **b** Measurement of PKG activity (high kinase activity corresponds to high OD measured photometrically) in lysate of Panc02 cells with a vehicle (PBS) (co) or with (DT3) 1 μM DT3, as described in detail in the "Materials and methods". The data are presented with SE and analyzed with the unpaired two-tailed t test, $*p < 0.05$ and $**p < 0.01$, ($n = 4–6$)

In this appalling situation, any attempt to search for new therapeutic modalities should be appreciated. Our in vitro data published recently have shown that inhibition of PKG could be a therapeutic option for PDAC (Karakhanova et al. 2014b). The aim of this study was to prove this hypothesis in vivo using a mouse model of PDAC.

This and our previous studies demonstrated that (i) the functional PKGI is expressed in PDAC cells, and (ii) there are cytotoxic effects of PKG inhibition operating through DT3. These features are not species dependent as both human and murine PDAC cells were sensitive to the DT3 treatment. It should be noted that IC50 of DT3 in the cell culture was in the same range in human and murine cells. Importantly, the cells of healthy liver were not sensitive to the cytotoxic effect of DT3. This allows us to suggest that DT3 could be considered as a potential anticancer drug after a careful in vivo examination. It should be stressed that the mechanism of DT3 cytotoxicity is a mixture of necrosis and late apoptosis.

In this work, we did not investigate toxicology, pharmacokinetic, and pharmacodynamic of DT3 in vivo. However, we observed no serious cytotoxic effects of DT3 when applied in dose based on our therapeutic schema. In agreement with our in vitro data, we found that the PDAC-bearing mice treated with DT3 developed smaller tumors in comparison with the control mice. Interestingly, while the invasion was not affected in vitro, the treated animals showed a trend to fewer colon metastases. At the moment, we cannot explain exactly this disagreement. However, the complexity of metastasis process involves among other such intricate pathways as epithelial-to-mesenchymal-transition (Zhu et al. 2014) and is strongly regulated by (tumor) micromilieu (Bazhin et al. 2013), which is not easy to re-modulate directly in vitro.

While our survival experiments did not show a survival benefit for the DT3-treated mice, this result indicates that although the tumor volume is one of the important tumor features, the survival of tumor-bearing animals does not exclusively depend on the tumor volume, and other parameters have to be taken into account. One extremely important point is that PDAC is characterized by very early dissemination (Haeno et al. 2012). In our work, we assessed only

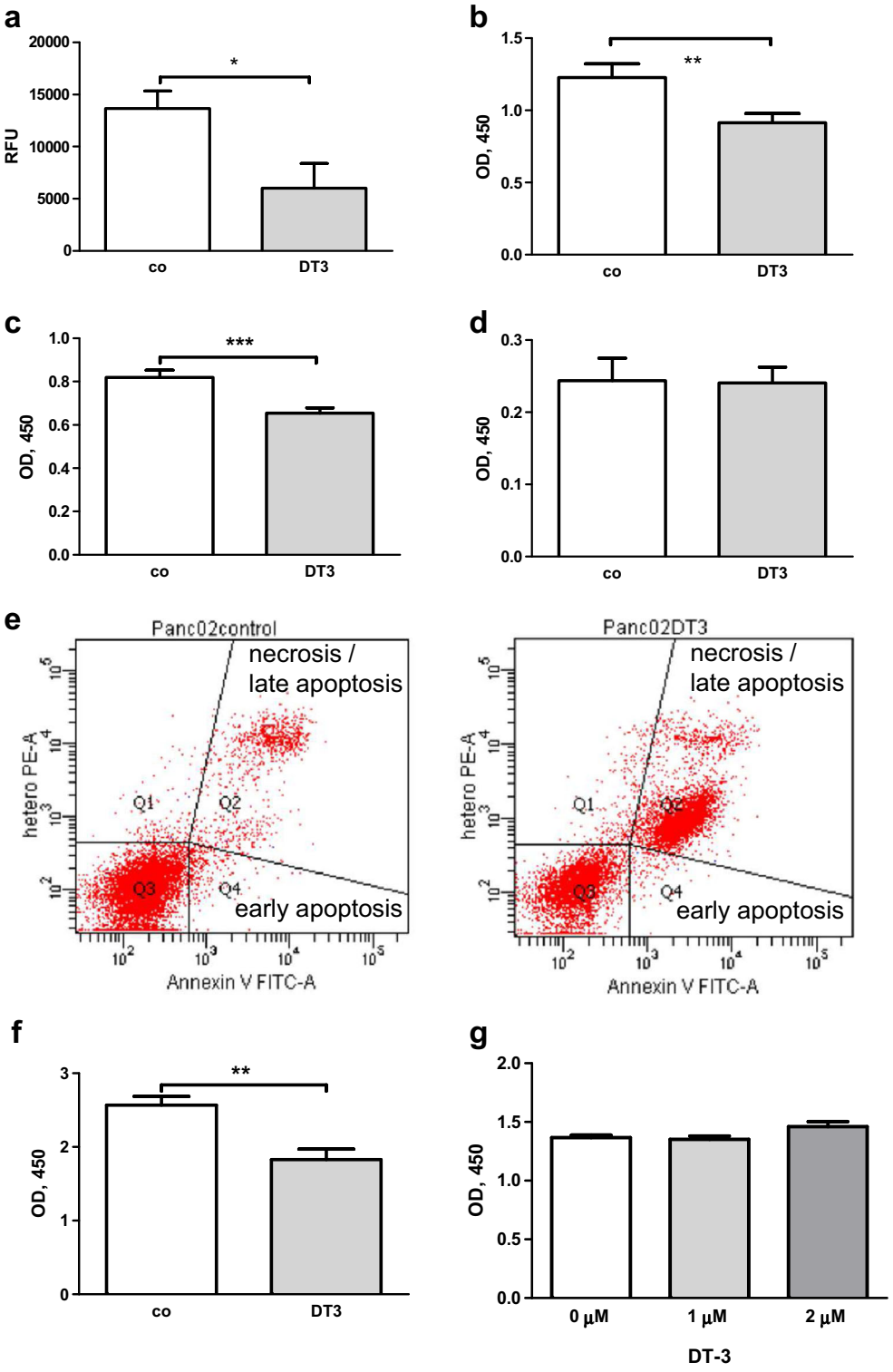

Fig. 2. DT3 induces cytotoxicity through necrosis and reduces proliferation and migration but not invasion of the Panc02 cell line. **a** Analysis of cell viability (high cell viability corresponds to high RFU measured fluorometrically) after 24-h incubation with 1 μM DT3 or with a vehicle control (PBS) (co). Analysis of cell proliferation (**b**), migration (**c**), and invasion (**d**) (high level of the tested parameters corresponds to high optical density (OD) measured photometrically) after 48-h treatment with DT3 by IC50 concentration (0.9 μM), or vehicle control (co). **e** Analysis of apoptosis and necrosis measured with flow cytometry; *Q2 quadrant* represents cell in necrosis and late apoptosis and *Q4 quadrant* shows cells in early apoptosis. Analysis of cell viability of the LPTA cells (**f) and of the** NCTC cells (**g**) after 24-h incubation with different (**g**) or 1 μM **(f)** concentrations of DT3. The data of four independent experiments are presented with SE and analyzed with the unpaired two-tailed t test (**c**), $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

macrometastases, but not micrometastases, which are normally present at a very early stage of the PDAC development. Another crucial point is the development of the treatment resistance. It is well-known that treatment of PDAC with chemotherapeutics acquits resistance which limits the therapeutic success (Li et al. 2004). Therefore, we cannot exclude that during the DT3 therapy, PDAC cells acquired resistance to this drug. Besides, with DT3, we could not achieve a complete tumor regression which can also be a reason for the negative result in survival experiments. All these options either alone or in combination could be responsible for the absence of survival improving by DT3 treatment.

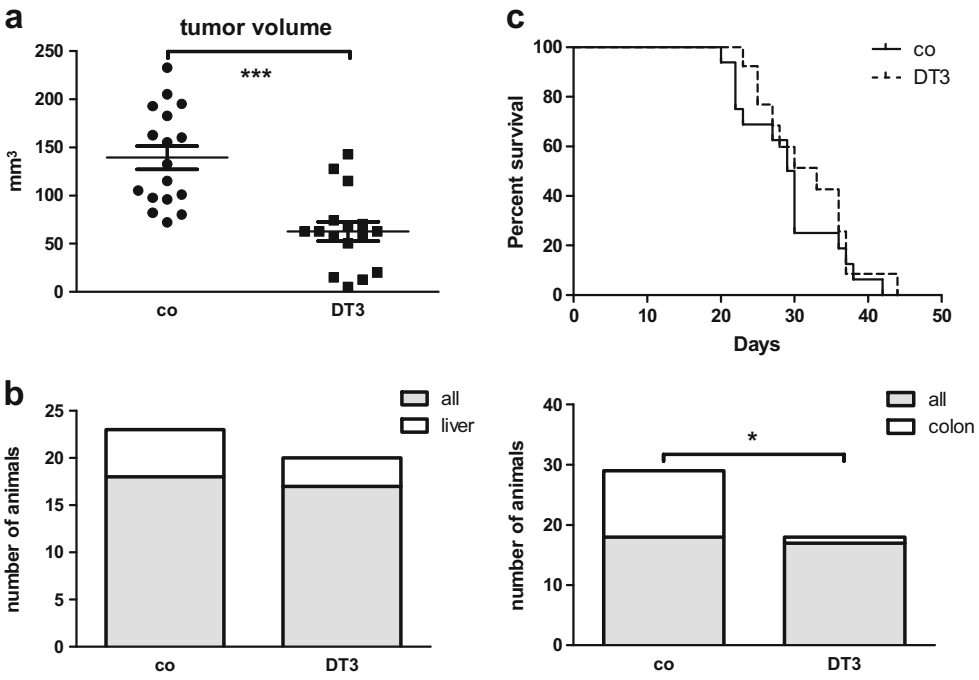

Fig. 3. In vivo effects of DT3 treatment of PDAC-bearing mice. **a** Tumor volume on day 21 after transplantation of Panc02, $n = 16–17$ mice per group; the data are presented with mean and SE and analyzed with the unpaired two-tailed t test, $***p < 0.001$. **b** Number of tumor-bearing animals with metastases, analyzed with $\chi 2$ method, $*p < 0.05$. **c** Survival analysis of DT3-treated and control vehicle-treated mice, analyzed with Kaplan-Meier curves with subsequent Log-rank test

In this respect, it is possible that we used with an underdose of DT3. Sure, the dose-escalation may lead to the development of toxicity and finally to adverse effects. Another possibility could depend on pharmacokinetics (distribution, metabolism, and excretion of DT3) or on pharmacodynamics (biochemical effects of DT3). Therefore, it is important in the future to try different schema of the DT3 treatment as well as to investigate pharmacokinetics and pharmacodynamics of DT3 to improve anti-cancer effects of this drug.

Another point should be discussed as well: in light of the above-mentioned study of Gambaryan and colleagues (Gambaryan et al. 2012), who showed an absence of the in vivo inhibition of PKGI by DT2, the question about the specificity of the in vivo DT3 effects arises. Both of the drugs (DT2 and DT3) are chemically related, and could have the same enzymatic effects. Therefore, in context of the signaling pathways investigated in this work, we cannot conclude that the inhibition of phosphorylation of GSK^{-3}, CREB, and P38

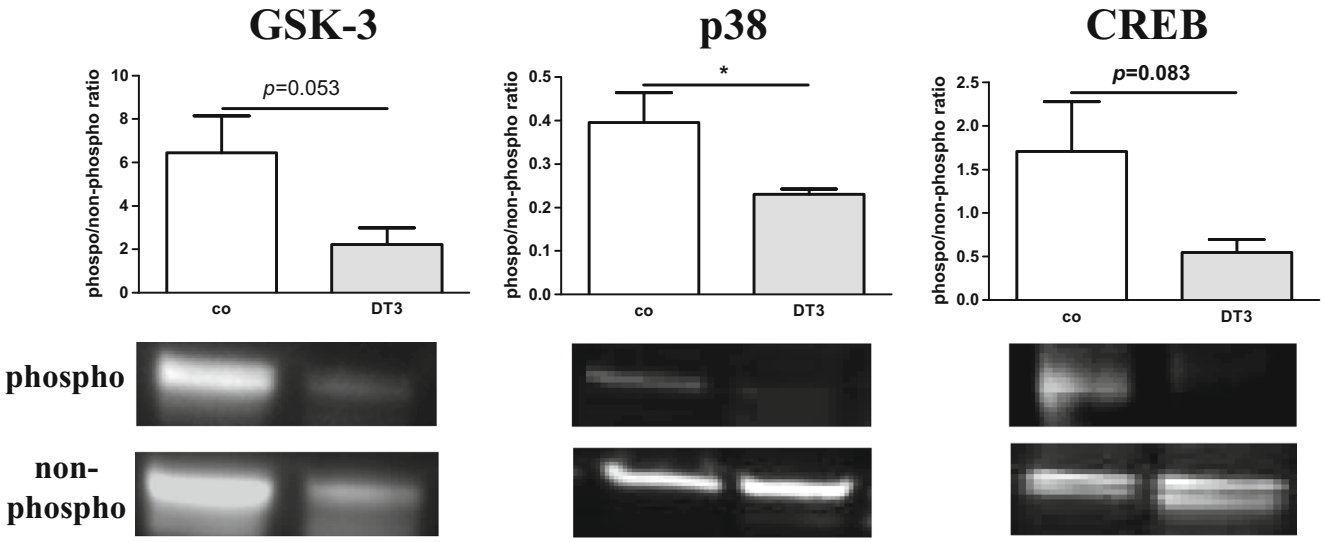

Fig. 4. DT3 inhibits phosphorylation of GSK−3, p38, and CREB. Panc02 cells were treated 24 h with 0.9 μM DT3. Afterwards, the live cells were analyzed with Western blot as described in "Materials and methods". Representative pictures of Western blotting membranes (for GSK−3, p38, and CREB) stained with antibodies specific for phosphorylated (phospho) or unphosphorylated (non-phospho) forms of the proteins tested in Panc02 as described in detail in "Materials and methods": *co* vehicle-treated (PBS) sample, D*T3* DT3-treated sample. The data from five animals in each group ($n = 5$) are presented with SE and analyzed with the unpaired two-tailed t test, $*p < 0.05$.

is due to the DT3-dependent inhibition of PKGI. This signaling profiling is extremely complex, and it cannot be excluded that these effects are due to the influence of DT3 on other kinases such as PKC, MAP kinases, and some others. However, in the oncological context, this point is irrelevant, if the drug manifests pronounced anticancer properties.

In conclusion, this work provides a "prove of principle" that DT3 irrespective of its pharmacological specificity could be important for the development of new strategies for the PDAC treatment.

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References

Bazhin AV, Schadendorf D, Owen RW, Zernii EY, Philippov PP, Eichmüller SB (2008) Visible light modulates the expression of cancer-retina antigens. Mol Cancer Res 6:110–118

Bazhin AV, Bayry J, Umansky V, Werner J, Karakhanova S (2013) Overcoming immunosuppression as a new immunotherapeutic approach against pancreatic cancer. Oncoimmunology 2:e25736. doi:10.4161/onci.25736

Bazhin AV, Shevchenko I, Umansky V, Werner J, Karakhanova S (2014) Two immune faces of pancreatic adenocarcinoma: possible implication for immunotherapy. Cancer Immunol Immunother 63:59–65. doi:10.1007/s00262-013-1485-8

Bosetti C, Bertuccio P, Negri E, La Vecchia C, Zeegers MP, Boffetta P (2012) Pancreatic cancer: overview of descriptive epidemiology. Mol Carcinog 51:3–13. doi:10.1002/mc.20785

Bryan NS, Bian K, Murad F (2009) Discovery of the nitric oxide signaling pathway and targets for drug development. Front Biosci 14:1–18

Cary SP, Winger JA, Derbyshire ER, Marletta MA (2006) Nitric oxide signaling: no longer simply on or off. Trends Biochem Sci 31:231–239. doi:10.1016/j.tibs.2006.02.003

Conroy T et al. (2011) FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 364:1817–1825. doi:10.1056/NEJMoa1011923

Dostmann WR, Taylor MS, Nickl CK, Brayden JE, Frank R, Tegge WJ (2000) Highly specific, membrane-permeant peptide blockers of cGMP-dependent protein kinase Ialpha inhibit NO-induced cerebral dilation. Proc Natl Acad Sci U S A 97:14772–14777. doi:10.1073/pnas.97.26.14772

Fraser M, Chan SL, Chan SS, Fiscus RR, Tsang BK (2006) Regulation of p53 and suppression of apoptosis by the soluble guanylyl cyclase/cGMP pathway in human ovarian cancer cells. Oncogene 25:2203–2212. doi:10.1038/sj.onc.1209251

Gambaryan S et al. (2012) The oligopeptide DT-2 is a specific PKG I inhibitor only in vitro, not in living cells. Br J Pharmacol 167:826–838. doi:10.1111/j.1476-5381.2012.02044.x

Haeno H, Gonen M, Davis MB, Herman JM, Iacobuzio-Donahue CA, Michor F (2012) Computational modeling of pancreatic cancer reveals kinetics of metastasis suggesting optimum treatment strategies. Cell 148:362–375. doi:10.1016/j.cell.2011.11.060

Karakhanova S, Golovastova M, Philippov PP, Werner J, Bazhin AV (2014a) Interlude of cGMP and cGMP/protein kinase G type 1 in pancreatic adenocarcinoma cells. Pancreas 43:784–794. doi:10.1097/MPA.0000000000000104

Karakhanova S et al. (2014b) Influence of interferon-alpha combined with chemo (radio) therapy on immunological parameters in pancreatic adenocarcinoma. Int J Mol Sci 15:4104–4125. doi:10.3390/ijms15034104

Li D, Xie K, Wolff R, Abbruzzese JL (2004) Pancreatic cancer. Lancet 363:1049–1057. doi:10.1016/S0140-6736(04)15841-8

Rosty C, Goggins M (2002) Early detection of pancreatic carcinoma. Hematol Oncol Clin North Am 16:37–52

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101–1108

Shevchenko I et al. (2013) Low-dose gemcitabine depletes regulatory T cells and improves survival in the orthotopic Panc02 model of pancreatic cancer. Int J Cancer 133:98–107. doi:10.1002/ijc.27990

Siegel R, Naishadham D, Jemal A (2012) Cancer statistics, 2012. CA Cancer J Clin 62:10–29. doi:10.3322/caac.20138

Warshaw AL, Fernandez-del Castillo C (1992) Pancreatic carcinoma. N Engl J Med 326:455–465

Zhu Y, Karakhanova, S., Huang, X., Deng, S., Werner, J., Bazhin AV. (2014) Influence of interferon-<alpha> on the expression of the cancer stem cell markers in pancreatic carcinoma cells. Exp Cell Res 323