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Evaluation of TAK-264, an Antibody-Drug Conjugate in Pancreatic Cancer Cell Lines and Patient-Derived Xenograft Models

Anna R. Schreiber¹, Anna Nguyen¹, Stacey M. Bagby¹, John J. Arcaroli^{1,2}, Betelehem W. Yacob¹, Kevin Quackenbush¹, Joe L. Guy³, Thomas Crowell⁴, Bradley Stringer⁴, Hadi Danaee⁴, Thea Kalebic⁴, Wells A. Messersmith^{1,2}, and Todd M. Pitts^{1,2,*}

¹Department of Medicine, Division of Medical Oncology, University of Colorado Anschutz Medical Campus, CO, USA;

²University of Colorado Cancer Center, Aurora, CO, USA;

³University of Michigan Comprehensive Cancer Center, Ann Arbor, MI, USA;

⁴Takeda, Cambridge, MA, USA

Abstract

Background: Antibody-drug conjugates (ADCs) are an emerging technology consisting of an antibody, linker, and toxic agent, which have the potential to offer a targeted therapeutic approach. A novel target recently explored for the treatment of pancreatic cancer is guanylyl cyclase C (GCC). The objective of this study was to determine the anti-tumorigenic activity of TAK-264, an investigational ADC consisting of an antibody targeting GCC linked to a monomethyl auristatin E payload *via* a peptide linker.

Methods: The antiproliferative effects of TAK-264 assessed in a panel of eleven pancreatic cancer cell lines. Additionally, ten unique pancreatic ductal adenocarcinoma cancer patient-derived xenograft models were treated with TAK-264 and the efficacy was determined. Baseline levels of GCC were analyzed on PDX models and cell lines. Immunoblotting was performed to evaluate the effects of TAK-264 on downstream effectors.

*Address correspondence to this author at the Department of Medicine, Division of Medical Oncology, University of Colorado Anschutz Medical Campus, 12801 E 17th Ave, MS 8117, Aurora, CO 80045, USA; Tel/Fax: 303-724-5951/303-724-3889; todd.pitts@ucdenver.edu.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethics Approval was obtained from the Institutional Animal Care and Use Committee (IACUC).

The humans study is in accordance with protocols approved by the Colorado Multiple Institutional Review Board (COMIRB).

HUMAN AND ANIMAL RIGHTS

Animals were used for the xenograft studies but they were approved by our IACUC.

All human reported experiments were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2008 (<http://www.wma.net/en/20activities/10ethics/10helsinki/>).

CONSENT FOR PUBLICATION

Consenting patients at the University of Colorado Hospital.

CONFLICT OF INTEREST

Potential conflict of interest are authors TK, BS, HD, TC who are employees of Takeda Limited. Takeda Pharmaceuticals partially funded this project and had no role in study design and decision to publish. The funders had no role in data collection and analysis or preparation of the manuscript. Authors ARS, AN, SMB, JJA, BY, KQ, JLG, WAM, TMP have no conflicts of interest.

Results: GCC protein expression was analyzed by immunoblotting in both normal and tumor tissue; marked increase in GCC expression was observed in tumor tissue. The *in vitro* experiments demonstrated a range of responses to TAK-264. Eight of the ten PDAC PDX models treated with TAK-264 demonstrated a statistically significant tumor growth inhibition. Immunoblotting demonstrated an increase in phosphorylated-HistoneH3 in both responsive and less responsive cell lines and PDAC PDX models treated with TAK-264. There was no correlation between baseline levels of GCC and response in either PDX or cell line models.

Conclusion: TAK-264 has shown suppression activity in pancreatic cancer cell lines and in pancreatic PDX models. These findings support further investigation of ADC targeting GCC.

Keywords

TAK-264; antibody-drug conjugate; pancreatic cancer; GCC; PDX models; p-histone H3

1. INTRODUCTION

Pancreatic adenocarcinoma continues to be one of the most deadly cancers with a 5-year survival rate of 8% [1]. Despite exciting advances in cancer therapy overall, pancreatic cancer is the fourth leading cause of cancer deaths in the United States annually [1]. Unless new therapies are developed, it is predicted that shortly before 2030, pancreatic cancer would become the second leading cause of cancer deaths, second only to lung cancer [2]. Current therapeutic options for advanced pancreatic cancer including gemcitabine combined with nab-paclitaxel, or fluorouracil, irinotecan, oxaliplatin and leucovorin (FOLFIRINOX), have provided only limited improvement in clinical outcome [3–5]. Therefore, novel treatments are needed to significantly impact survival in patients with this disease.

Antibody-drug conjugates (ADC) are an exciting class of anticancer agents, which have the potential to offer a targeted therapy with an increased therapeutic index, compared to conventional chemotherapy regimens. ADCs consist of an antibody linked to a cytotoxic agent that specifically binds to target antigens expressed on tumor cells [6]. Consequently, toxicity could be minimized with the ADC delivery system as toxic drug metabolites are released only inside target expressing tumor cells [7]. Currently, ADC therapies for relapsed CD-33 positive acute myeloid leukemia, refractory or relapsed Hodgkin's lymphoma, and relapsed systemic anaplastic large-cell lymphoma and HER-2 positive breast cancer have been approved for use by the Food and Drug Administration (FDA) [8–11]. There is a growing evidence that ADCs have the ability to provide meaningful clinical benefit, but clinical trial results largely varied [8, 12]. Challenges to ADC development include target specificity, optimal linkers and toxic payloads, all of which impact response/toxicity, and resistance mechanisms. Their potential in pancreatic cancer is for the most part uncharted.

TAK-264 consists of the antibody conjugated to mono-methyl auristatin E (MMAE), a potent inhibitor of microtubule polymerization, *via* a maleimido-caproyl-valine-citrulline peptide linker [9]. TAK-264 utilizes a fully human IgG1 ADC, which selectively targets guanylyl cyclase C (GCC). Upon binding to GCC, TAK-264 is internalized into the cell where the linker is cleaved, allowing the cytotoxic agent MMAE to be released. MMAE then binds to microtubules in the cell, halting the cell cycle and resulting in apoptosis [13]. GCC

is a transmembrane G protein receptor found on the apical surface of intestinal enterocytes [14]. In the intestines, GCC is involved in the transfer of fluids, plays a role in the cytostasis of epithelial cells of the colon and may contribute to inflammation in inflammatory bowel disease [15]. GCC is highly expressed in colorectal carcinomas as well as adenocarcinomas of the upper GI tract [16, 17]. In preclinical studies, TAK-264 has shown strong antitumor activity in metastatic colorectal carcinoma (mCRC) xenograft models and has also shown antitumor activity in GCC expressing pancreatic cancer xenograft models [17–19].

In this study, we used patient-derived pancreatic tumor xenograft models to further explore the antitumor efficacy and anti-tumor mechanisms of TAK-264 [20].

2. METHODS

2.1. Chemicals and Reagents

TAK-264 was provided by Takeda Pharmaceuticals (Cambridge, MA). The peptide linker and MMAE toxin were licensed from Seattle Genetics. For *in vivo* studies, TAK-264 was suspended in saline to a concentration of 2.5mg/mL and dosed at 10mg/kg, (IV), QW. For *in vitro* studies, TAK-264 was diluted into culture media and dosed appropriately for each experiment.

2.2. Cell Lines and Culture

Human pancreatic cancer cell lines were obtained from ATCC. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) or Roswell Park Memorial Institute (RPMI) medium supplemented with 15% FBS and 10–20 units/mL of insulin. Both mediums contained 1% nonessential amino acids and 1% penicillin/streptomycin. Cells were cultured at a temperature of 37°C with an atmosphere of 5% CO₂. Cells were screened for the presence of Mycoplasma (Mycoplasma Detection Kit; BioTool, Houston, TX). All cell lines were tested and authenticated by the University of Colorado Cancer Center DNA Sequencing and Analysis Core [21].

2.3. Proliferation Assay

Proliferation effects of TAK-264 on cell lines were analyzed using a Sulforhodamine B (SRB) assay. Cells were aliquoted into 100µl per well suspensions containing 3,000 to 5,000 viable cells and plated onto 96-well flat-bottomed plates overnight. Cells were then treated with TAK-264 for 72 hours. Following treatment, cell medium was discarded and the cells were fixed with cold 10% TCA (trichloroacetic acid) for 30 minutes at 4°C. Following this, cells were then washed with water and then stained at room temperature with 0.4% SRB (Fisher Scientific) for 30 minutes. Finally, the cells were washed with 1% acetic acid and then treated with 10 mmol/L Tris solution to allow the stain to solubilize. Using a Biotek Synergy 2 96-well plate reader, the plates were read at an absorbance wavelength of 565 nm. Using raw absorbance data, cell proliferation curves were formulated [21].

2.4. Cell Cycle Analysis

MIA PaCa-2 and PANC-1 cells were plated with 2.5×10^5 cells per well onto 6-well plates and were incubated overnight. Cells were then treated with 12.5µg/mL of TAK-264 for 24

hours. Culture media was then removed, the cells were washed with 1X PBS and then were collected. The cells were centrifuged at 1,500 rpm and then resuspended in Krishan's stain. Cells were placed at 4°C for 24 hours. The next day the cells were analyzed at the University of Colorado Cancer Center Flow Cytometry Core Facility using a Gallios flow cytometer [22, 23].

2.5. GCC shRNA Knockdown

GCC shRNAs were purchased from Origene and stable clones were generated. Following the manufacturer's recommendations, MIA PaCa-2 cells were incubated in a 6 well plate overnight and then transfected with 1µg of each of the shRNA plasmids using Xfect Single Shots transfection reagent (Clontech) [24]. Seventy-two hours after transfection, knockdown cells were selected using 2.0µg/mL of puromycin. Knockdown of targeted genes was confirmed by semiquantitative RT-PCR and western blot analysis [25].

2.6. Pancreas Explant Xenograft Model

Patient derived pancreatic tumor specimens and adjacent normal samples were obtained at the time of surgery from consenting patients at the University of Colorado Hospital in accordance with protocols approved by the Colorado Multiple Institutional Review Board (COMIRB). Approval was obtained from the Institutional Animal Care and Use Committee (IACUC), and four to six week old female athymic nude mice were purchased from Envigo (Formerly Harlan laboratories, Indianapolis, IN).

Tumor pieces (~3mm³ fragments) were injected into the right and left flank of nude mice through use of a trochar. Each treatment group contained 5–6 mice with tumors injected into the right and left flank to give ~10 evaluable tumors. Mice were randomized into control or TAK-264 groups when tumor volumes reached ~150–300 mm³. Mice were treated weekly with TAK-264 (10mg/kg) by IV tail injections for at least 17 days. Mice were evaluated every day for evidence of toxicity and illness. Tumor size was evaluated twice each week by caliper measurements using the following formula: tumor volume = (length x width)² × 0.52 [26].

2.7. Immunoblotting and Immunohistochemistry (IHC)

Tumor (15 mg/tumor tissue) tissues were homogenized using a Qiagen tissue lyser and protein lysates were obtained by centrifuging at 16,000 g at 4°C for 10 min. A 660 Protein Assay kit (Thermo Fisher) was used to determine the total protein concentration per sample. Forty micrograms of the sample were electrophoresed on 4–12% Bis-Tris precast gels (Life Technologies, Carlsbad, CA) and transferred to a nitro-cellulose membrane using the iBlot (Life Technologies, Carlsbad, CA). At room temperature, membranes were blocked with PBS with 1% casein for one hour. The primary antibodies (Cell Signaling Technology) were diluted to 1:1,000 in blocking buffer made up of 0.1% (v/v) Tween 20 and were added to the membranes which were placed on a rocker and incubated overnight at 4°C. Following overnight incubation, the membranes were washed three times with TBST and then incubated with either anti-rabbit or anti-mouse IgG DyLight antibodies (Cell Signaling Technology) for 1 hour at room temperature. Blots were then read using an Odyssey Imager (Licor, Lincoln, NE) [27].

IHC analysis of GCC expression was performed using anti-GUCY2C antibody from Abcam using manufacturer instructions.

2.8. Statistical Analysis

A paired t-test was used to examine the differences between matched normal and pancreatic tumor tissues. All samples were normalized to actin levels prior to statistical testing. For the PDX models, we performed an unpaired Student t-test to determine whether the means between control and TAK-264 were significant (p-value were <0.05) at end of treatment. The error bars (standard error of the mean) are shown in the graphs. All statistical tests were carried out by GraphPad Prism software version 5.0c (La Jolla, CA).

3. RESULTS

3.1. Assessment of GCC Expression in Normal vs. Pancreatic Tumor Tissue

We first investigated if there are differences in GCC expression among matched normal and pancreatic tumor tissues obtained from patients. A total of six patient-matched normal and tumor tissues were evaluated by GCC immunoblotting. As shown in (Fig. 1), pancreatic tumor tissues were found to have a markedly higher GCC expression than matching normal tissue ($p < 0.001$).

3.2. Anti-proliferative Effects of TAK-264 Against Pancreatic Cancer Cell Lines

After determining that GCC expression is significantly increased in pancreatic tumor tissues, as compared to normal tissue, we assessed whether targeting the GCC receptor with TAK-264 would cause antiproliferative effects in pancreatic cancer cell lines. Eleven pancreatic cancer cell lines were treated with TAK-264 (dose range 0.4 – 25 $\mu\text{g/ml}$) for 72 hours and analyzed by a SRB proliferation assay. As demonstrated in (Fig. 2), five pancreatic cancer cell lines were deemed more responsive to TAK-264 (L3.6, MIA PaCa-2, Panc 03.27, Panc 05.04 and Panc 02.03), as proliferation was less than 50% after treatment with 25 $\mu\text{g/mL}$ of TAK-264. In contrast, six cell lines were found to be less responsive as proliferation exceeded 50% after treatment with 25 $\mu\text{g/mL}$ of TAK-264. A peak concentration of 25 $\mu\text{g/mL}$ was selected based on pharmacokinetic data demonstrated in the Phase I studies of TAK-264 [13].

3.3. TAK-264 Treatment Effects on Cell Cycle and Apoptosis

To delineate whether the anti-proliferative effects of TAK-264, were a result of cell cycle arrest or apoptosis, we treated the MIA PaCa-2 (more responsive) and PANC-1 (less responsive) cell lines with 12.5 $\mu\text{g/ml}$ of TAK-264 for 24 hours. While cell cycle arrest was not observed in the MIA PaCa-2 cell line (Fig. 3A), the PANC-1 cell line demonstrated a decrease in G1 and an induction of a G2/M arrest when compared to the untreated control ($p=0.06$) (Fig. 3B). An apoptosis assay was performed at 8 and 24 hours and no induction of apoptosis was noted (**data not shown**). Furthermore, we observed no differences in the activation of p53 and Chk-2, key regulators of cell cycle and apoptosis after exposure of TAK-264 (12.5 $\mu\text{g/ml}$) in the MIA PaCa-2 and PANC-1 cell lines (**data not shown**). In contrast, an increase was seen in p-histone H3 following treatment with TAK-264 (Fig. 3C and D).

3.4. Assessment of TAK-264 Efficacy in GCC Knockdown Cells

To explore the relationship between GCC expression and sensitivity to TAK-264, shRNA GCC knockdown studies were conducted in the MIA PaCa-2 cell line. MIA PaCa-2, scramble control (MIA PaCa-2), and MIA PaCa-2 GCC knockdown cell lines were exposed to TAK-264 at concentrations 0.4 – 25 µg/ml for 72 hours and % proliferation was assessed by a SRB assay. As shown in (Fig. 4), the MIA PaCa-2 GCC knockdown cell line responded less to TAK-264 when compared to the scramble control and MIA PaCa-2 cell lines at 12.5 and 25 µg/ml of TAK-264.

3.5. Efficacy of TAK-264 in Patient-derived Xenograft Models

To evaluate the efficacy of TAK-264 in mouse models of pancreatic cancer, ten pancreatic PDX models were treated with 10mg/kg of TAK-264 for at least 17 days. As depicted in (Fig. 5), a significant decrease in tumor growth was identified in 8 out of 10 pancreas cancer PDX models (Panc122, 150, 193, 266, 268, 269, 272, 277) treated with TAK-264. The two models that were less sensitive to TAK-264 included Panc129 and Panc137.

Expression of GCC was evaluated in all the pancreatic PDX models by IHC to determine whether there was an association between GCC expression and response to TAK-264. As illustrated in (Fig. 5), all pancreas tumors exhibited a marked baseline GCC expression, including those which did not respond to treatment with TAK-264 and treatment did not affect the GCC expression.

3.6. In-vivo Assessment of TAK-264 Effects by Immunoblotting

Highly responsive PDX tumor Panc 150 and low responsive PDX tumor Panc 137, were compared for expression of downstream effectors. In both models, higher expression of p-Histone H3 was observed in TAK-264 treated cells when compared to untreated controls. This indicates inhibition of tubulin polymerization and consequent apoptosis of treated cells (Fig. 6). Panc 150 and Panc 137 have not shown significant changes in expression of p-Chk2 nor p53 when compared to control (**data not shown**).

4. DISCUSSION

ADCs are an evolving field of oncologic research with great potential to aid in the treatment of deadly malignancies. This preclinical study evaluated TAK-264, a novel ADC targeting GCC, and its potential use as a pancreatic cancer agent. TAK-264 was used as a single agent and was explored in both *in vitro* pancreatic cancer cell lines and *in vivo* pancreatic tumor mouse explant models. The results demonstrate that the level of GCC expression does not appear to correlate with a level of treatment response.

Multiple studies have indicated that GCC expression is upregulated in pancreatic cancer when compared to normal pancreas tissue [28–30]. GCC is expressed on the apical side of normal tissues, however in tumors the cell polarity and structure are altered. This change of cellular localization is assumed to allow GCC targeted therapies to specifically affect malignant cells, when administered intravenously, without affecting normal tissue [5]. We have found a marked increase in GCC expression in tumor tissue when compared to normal

tissue. This is consistent with a recent study also showing that normal pancreatic tissue, does not express GCC [31].

Similarly, a recent study examining the cell cycle effects of taxane treated lung cancer cells demonstrated a G2/M arrest and decrease in G1 following treatment, which is consistent with our findings. Both TAK-264 and taxanes, impact polymerization of microtubules, resulting in cell death [32, 33]. When examining the MIA PaCa-2 cell line, no cell cycle arrest was noted. Paclitaxel in human carcinoma and leukemia cell lines has been found to produce, in a dose-dependent fashion, a greater accumulation at G2/M and a steady increase of condensed nuclei after 24 hours [34].

In this study, we have observed that both cell lines and tumor models exhibited an increase in expression of DNA damage indicator p-Histone-H3 following a treatment with TAK-264. The phosphorylation of histone H3 has been found to be a necessary step in the process of cell division [35, 36]. The increase in p-Histone-H3 is a result of the toxic agent MMAE inducing damage of tumor cells by halting tubulin polymerization which prompts apoptosis of cells [37]. Other agents such as vinblastine, podophyllotoxin and nocodazole have been found to assert their lethal effects by a similar mechanism of action. Specifically, these agents depolymerize microtubules resulting in death to the affected cells [38]. A recent study comparing the treatment of HeLa cells with nocodazole and a novel tubulin inhibitor STK899704, found that the treatment of both agents induced a rise in p-histone H3 [39]. This is similar to our findings which demonstrated that inhibition of microtubule polymerization damages the cells and increases p-histone H3.

Our pre-clinical findings parallel results observed in clinical studies of TAK-264 [5, 13]. In those studies GCC expression did not correlate with antitumor activity [13]. The drug exhibited a manageable safety profile, however, GCC expression levels did not correlate with clinical outcome. This suggests a further need for additional research which could lead to optimizing the individual components of the ADC including mAb, linker and toxin. Also a potential failure of the ADC to internalize needs to be better examined [5]. We observed that levels of GCC expression did not correlate with a level of response in PDX models. However, our findings show that a decrease in proliferation of GCC knockdown cells is inhibited but only at high concentrations of TAK-264. This suggests that GCC is necessary to mediate response to TAK-264. Our data demonstrated that GCC was highly expressed at baseline and following treatment in all tumor samples derived from human pancreatic carcinomas, which could explain why all 10 PDX models showed some degree of stabilization to TAK-264. However, there was no tumor shrinkage in any of the models tested.

Our results are comparable to an earlier study which determined the effects of TAK-264 in colorectal tumors and HEK293-GCC cell lines [31]. That study demonstrated that despite GCC expression, certain models were poorly responsive to TAK-264. An earlier study reported that TAK-264 was found near the tumor surface 7 days after administration, suggesting that the ADC was able to reach the tumor cells. Additionally, that study demonstrated that in both resistant and sensitive models free MMAE was detected inside the cells, thus indicating that the ADC was likely internalized or processed in both models. The

expression of drug-efflux pumps such a P-glycoprotein (PGP) was also examined, however when mRNA levels were compared there was no difference in sensitive and resistant models. Similarly, when PGP and ABCB1 levels were examined in all PDX models, no significant differences were observed (Average sensitive 22.3 RPKM, Average resistant 14.8 RPKM). We can speculate that molecular factors involved in a regulation of sensitivity to MMAE could be driving response to treatment. Also the factors which are involved in cleavage of the linker could impact the effects of TAK-264 [40]. However, expression of cathepsin B was analyzed in all PDX models and no significant differences were noted when comparing more responsive tumors to less responsive tumors.

CONCLUSION

Our study demonstrated that TAK-264 has the ability to target GCC and cause DNA damage in pancreatic carcinoma models. Supporting a hypothesis that further investigation of GCC as a target for novel therapeutic modalities could be of interest. However, our data show that the extent of tumor and DNA damage does not correlate with GCC expression. While ADCs have a potential to offer effective therapeutic, there are also challenges. Innate or acquired resistance is frequently encountered and could potentially explain a lack of correlation between GCC and TAK-264 response observed in our study and previously published studies [31, 41]. ADCs are complex therapeutic agents that could offer high efficacy when all three components (mAb, linker and cytotoxin) are optimized and a patient population is properly selected. Due to the limited clinical efficacy that was observed with TAK-264, MMAE could be replaced with a more potent toxin to provide a more effective ADC. Efforts will depart from further evaluating TAK-264 to developing a novel ADC targeting GCC.

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Fig. (1).
Baseline immunoblotting with anti-GCC antibodies in tumor and matching normal samples.

Fig. (2). Proliferation analysis of 11 pancreatic cancer cell lines. Cells were treated with increasing amounts of TAK-264 and evaluated for proliferation *via* SRB assay as described in Methods. Cell lines were deemed more responsive if proliferation was less than 50% after treatment with 25μg/mL of TAK-264 and were deemed less responsive if proliferation exceeded 50% after treatment with 25μg/mL of TAK-264.

Fig. (3).

Two PDAC cell lines were treated for 24 hours with 12.5 $\mu\text{g/mL}$ of TAK-264, stained with propidium iodide and analyzed by flow cytometry for cell cycle arrest (**A,B**). PDAC cell lines were treated as described above and immunoblotting performed to determine levels of P-Histone H3, indicating tubulin polymerization following treatment (**C,D**).

Fig. (4). Proliferation assay of scramble control *versus* GCC shRNA knockdown in TAK-264 treated MIA PaCa-2 cells (A). Western blot analysis confirming knockdown of GCC (inset), (B).

Fig. (5). Growth curves of 10 PDX models treated with 10mg/kg of TAK-264 and vehicle control. TAK-264 was administered once a week IV for at least 17 days. A t-test analysis was performed on vehicle *versus* treated tumors ($p < 0.05$ annotated * and $p < 0.001$ annotated **). GCC IHC analysis was performed at end of study (EOS) on the vehicle control and TAK-264 treated.

Fig. (6).
Immunoblotting of PDAC PDX tumors PANC150 (responsive) and PANC137 (non-responsive) showing expression of p-Histone-H3.