A combination of DR5 agonistic mAb with gemcitabine targets pancreatic cancer stem cells, and results in long-term disease control in human pancreatic cancer model

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Abstract

Pancreatic ductal adenocarcinoma (PDA) is an aggressive malignancy with one of the worst outcomes among all cancers. PDA often recurs after initial treatment to result in patient death despite the use of chemo-/radiation therapy. PDA contains a subset of tumor-initiating cells capable of extensive self-renewal known as cancer stem cells (CSCs), which may contribute to therapeutic resistance and metastasis. At present, conventional chemotherapy and radiotherapy are largely ineffective in depleting CSC pool, suggesting the need for novel therapies that specifically target the cancer-sustaining stem cells for tumor eradication and to improve the poor prognosis of PDA patients. In this study, we report that death receptor-5 (DR5) is enriched in pancreatic CSCs compared to the bulk of the tumor cells. Treating a collection of freshly generated patient-derived PDA xenografts with gemcitabine (GEM), the first-line chemotherapeutic agent for PDA, is initially effective in reducing tumor size, but largely ineffective in diminishing the CSC populations, and eventually culminated in tumor relapse. However, a combination of tigatuzumab, a fully humanized DR5 agonist mAb, with GEM prove to be more efficacious by providing a double hit to kill both CSCs and bulk tumor cells. The combination therapy produced remarkable reduction in pancreatic CSCs, tumor remissions and significant improvements in time to tumor progression in a model which is considered more difficult to treat. These data provides the rationale to explore the DR5 directed therapies in combination with chemotherapy as a therapeutic option to improve the current standard of care for pancreatic cancer patients.
Introduction

Pancreatic ductal adenocarcinoma (PDA) is among the most lethal human cancers and its incidence is rising in the United States (1). Resistance to chemotherapy is thought to be a major cause of treatment failure in PDA patients (2, 3). As our understanding of PDA evolves, increasing evidence is growing to support a role for tumor-initiating cells called cancer stem cells (CSCs) in this devastating disease (4). Recent studies suggest that PDA is driven by a small population of CSCs that are responsible for tumor initiation and propagation (5, 6). At present, conventional chemotherapy and radiotherapy affect rapidly dividing PDA cells that constitute the tumor bulk, thus reducing tumor mass but probably fail to target CSCs that drive tumorigenesis and metastasis, which might be responsible for treatment failure and tumor recurrence in many patients (7). Although the clinical relevance of CSCs beyond experimental models is still lacking, the high frequency of relapse after conventional cytotoxic chemotherapies in PDA suggest that CSCs survive standard treatments (8).

Decades of efforts have witnessed the failure of many chemotherapeutic regimens tested in PDA and the current standard of care chemotherapeutic agent, gemcitabine (GEM), extends patient survival by only a few weeks (9). In the last 20 years, a large number of patients have been treated in randomized, large phase III clinical trials, but results have been globally disappointing (10). A marked change in treatment paradigm is essential to move beyond the persistently dismal outcome for the majority of PDA patients (11). It is becoming evident that a cancer treatment that fails to eliminate CSCs may allow the regrowth of the tumor (12). Recent reports indicate that a subpopulation of PDA cells functionally resembling CSCs have strong resistance to GEM both in vitro and in vivo (13, 14). In addition, treatment with ionizing radiation and GEM resulted in the enrichment of CSC populations in human primary PDA xenografts (15, 16). For these
reasons, targeting cancer-sustaining stem cells might be an attractive strategy for more effective cancer treatment.

In the quest to discover antitumor agents with greater specificity and potency, efforts have been directed toward developing monoclonal antibodies that recognize antigens unique to or overexpressed by cancer cells. Tumor necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL) and its agonistic antibodies, which are being evaluated clinically as anticancer therapies, selectively kill cancer cells through the death receptors DR4 and DR5 (17, 18). Importantly, purified recombinant human TRAIL suppresses tumor growth and showed little or no overt toxicity when systemically administered to animals (19). DR5 expression has been detected with high frequency in tumor cell lines and clinical tumor specimens (20). Cancer cell lines express DR5 more frequently than DR4 and studies demonstrated that DR5 might contribute more than DR4 to TRAIL-induced apoptosis in cancer cells that express both death receptors (21). DR5 levels have been reported to be elevated in primary PDA tissues as compared to the normal pancreas (22). A novel murine anti-human DR5 mAb, TRA-8, has been reported to induce apoptosis in several tumor cell lines and inhibited the growth of tumors xenografted in mice (23, 24). Tigatuzumab, a humanized version of TRA-8, is currently in clinical trials as a therapy for solid tumors (25). Tigatuzumab has selective toxicity toward tumor cells expressing DR5 and showed robust antitumor efficacy in human malignancies without damage to other tissues or hepatocyte cytotoxicity (26).

In the present study, we investigated the in vivo efficacy of tigatuzumab monotherapy and in combination with GEM in a panel of pancreatic cancer xenografts, which were generated from PDA patients. We provide evidence for the first time that pancreatic CSCs are enriched with DR5. GEM, the first-line agent for PDA, is initially effective in reducing tumor size, but not capable of reducing tumorogenic CSCs, and eventually culminated in tumor recurrence. However, combination of GEM with tigatuzumab markedly reduced pancreatic CSCs and subsequently prevented tumor recurrence. The present findings are supportive of the notion that targeting CSCs as an attractive avenue
in cancer treatment and provides the rationale to explore DR5 directed therapies in combination with chemotherapy.

**Materials and Methods**

**Animals and establishment of xenografts model.** Female *nu/nu* athymic mice (Harlan) were used for the study. Animals were maintained under pathogen-free conditions and a 12 h light-dark cycle. Animal experiments were conducted following approval and in accordance with the Animal Care and Use Committee guidelines of the Johns Hopkins University. Fresh pancreatic tumor pieces obtained from patients at the time of surgery, with informed written patient consent, were implanted subcutaneously (s.c.) into the flanks of 6-week-old mice. The patients had not undergone chemotherapy or radiation therapy before surgery. Grafted tumors were subsequently transplanted from mouse to mouse and maintained as a live PancXenoBank according to an IRB approved protocol (27).

**In vivo tumor therapy studies.** To establish the efficacy of tigatuzumab, GEM and the combination of GEM with tigatuzumab, tumors from eight separate patient xenografts were implanted (s.c.) into the flanks of athymic mice. Cohorts of mice with tumor size of ~200 mm³ in each xenografts were randomized to four treatment groups (6 mice; 10 tumors/group): (a) vehicle (control); (b) tigatuzumab 3 mg/kg (i.v) once weekly for 4 weeks; (c) GEM 100 mg/kg (i.p) twice a week for 4 weeks and (d) GEM plus tigatuzumab in the above mentioned dose and frequency for 4 weeks. Tigatuzumab (Daiichi Sankyo Co., Ltd.) and GEM (Eli Lilly and Company) doses were selected based on previously published reports (26, 28). Animals were sacrificed after the final dose of drug treatment (28th day) except 6-8 tumors in GEM and combination treatment group, which were followed for up to 120 days to investigate whether the combination therapy results in a durable tumor inhibition. Tumor size was evaluated twice per week by caliper measurements. Tumor volume was calculated using the formula: tumor volume = [length
Relative tumor growth (T/C) rate was calculated using the formula: (mean tumor volume of drug-treated group / mean tumor volume of control group) x 100. Tumor doubling time was calculated as the time elapsed from the day of first treatment to double the initial tumor size on an individual tumor basis and analyzed using a log-rank test based on the Kaplan-Meier method.

Protein extraction and western blot analysis. Protein extracts were prepared from tumors according to previously published methods (29). Briefly, tumors from two separate animals of control and treatment groups (on day 28) were minced on ice in prechilled lysis buffer. The minced tissue was homogenized using a Dounce homogenizer and centrifuged at 16,000 x g at 4°C for 10 min. Protein lysates (30 μg) were fractionated by SDS-PAGE, electrotransferred to nitrocellulose membranes, blotted with primary antibodies for TRAIL (Santa Cruz Biotechnology Inc.), DR5, Fas, FADD, TRADD, Pro and cleaved Capase-8, Pro and cleaved Capase-3, Bid, Bax, Cleaved PARP, XIAP, p-53 and β actin (Cell Signaling Technology). TRIAL and Caspase-8 were mouse mAb’s and the other antibodies used were rabbit monoclonal/polyclonal origin. After washing thrice with Tris-buffered saline (TBS, the membranes were incubated for 1 h at room temperature with horseradish peroxidase–conjugated secondary antibodies, rabbit, or mouse IgG-horseradish peroxidase (Santa Cruz Biotechnology Inc.). After washing thrice with TBS, antibody binding were detected by enhanced chemiluminescence (GE Healthcare) as previously reported (29). Band intensities were quantitated by densitometric scanning.

Cancer stem cell quantification and DR5 expression by flow cytometry. Tumors grown subcutaneously in nude mice were harvested and single cell suspensions were generated by mincing tumors using sterile razors, followed by incubation in dispase and collagenase type IV (both from Sigma) at 37°C for 2 h with agitation. Debris was removed by passing the cell suspension through a 70-micrometer filter (BD Biosciences) and further purified by density centrifugation using Ficoll-Paque Plus (GE Healthcare). The purified cells were washed twice in cold Dulbecco’s modified Eagle medium. Cells were incubated with ALDEFLUOR reagent (Stem Cell Technologies) in the presence or
absence of diethylamino-benzaldehyde (DEAB) following the manufacturer’s protocol. Cells were then stained for 15 minutes at 4°C with different combinations of the following antibodies: anti-mouse CD31-biotin (BD Biosciences), mouse lineage cocktail-biotin (Miltenyi Biotec), and anti-mouse H-2K^d-biotin (BD Biosciences), anti-human CD44-allophycocyanin (APC) (BD Biosciences; clone G44-26), anti-human CD24-phycoerythrin (PE) (BD Biosciences; clone ML5), anti-human CD24-fluorescein isothiocyanate (FITC) (BD Biosciences; clone ML5), anti-human DR5-PE (eBiosciences; clone DJR2-4), mouse specific IgG2b κ-APC, mouse specific IgG2a κ-PE, and mouse specific IgG1 κ-PE. The cells were washed and then incubated with streptavidin-peridinin chlorophyll (PerCP) for 10 minutes at 4°C. Finally, the cells were washed once again and resuspended in ALDEFLUOR buffer containing 2 μg/mL propidium iodide. The cells were analyzed using a FACS Aria Flow Cytometer (BD Biosciences). The cells were gated based on forward scatter and side scatter properties followed by exclusion of mouse derived and non-viable cells. ALDEFLUOR^+, CD24^+CD44^+ and DR5^+ tumor cells were determined based on the fluoresce as previously reported (30). In a separate experiment, Panc219 tumor bearing mice were treated with tigatuzumab, GEM, and GEM plus tigatuzumab in the mentioned doses and frequencies for four weeks. Tumors were harvested and stem cell populations were purified and analyzed using a FACSAria Flow Cytometer as described above.

**Immunohistochemical detection of CD24.** Tumor tissues from the saline treated and other treatment groups on day 28 were fixed in formalin and processed into paraffin blocks. Two tumors per treatment group were analysed. Sections were deparaffinized in xylene and rehydrated in graded alcohol washes. Antigen retrieval was performed by incubating the slides in boiling sodium citrate buffer (10 mM, pH 6.0) for 30 minutes, and endogenous peroxidases were quenched by incubating the slides in 3% hydrogen peroxide in methanol for 10 minutes at room temperature. The slides were then incubated with CD24 mouse monoclonal antibody (Clone SN3b; Thermo Scientific Inc.). Envision plus dual-link polyper–horseradish peroxidase (DAKO) was used as the detection chemistry, and 3,3’-diaminobenzidine as the chromagen. After immunostaining, slides
were counterstained with haematoxylin. The staining was scored as the number of CD24+ tumor cells (membranous and/or cytoplasmic staining for CD24) over total tumor cells (0-100%) multiplied by staining intensity. We used a 0 to 3 scale for staining intensity: 0, completely negative; 1, weak positivity; 2, moderate positivity; 3, strong positivity (16, 28). CD24 staining was scored in approximately 10 random fields with a minimum of 1,000 tumor cells by a pathologist in a blind way.

**Quantitative real-time reverse transcription-polymerase chain reaction.** RNA was purified from the Panc219 and Panc410 xenografts on day 28 using the RNeasy kit (Qiagen). Five micrograms of total RNA from two separate tumors in each group were used for cDNA synthesis and reverse transcription was performed using Superscript II (Invitrogen) per standard protocol. Quantitative PCR was performed on a MyIQ real-time PCR machine (BioRad) and using TaqMan primer/probe sets (Applied Biosystems) for β-actin, ALDH1A1 (Hs00167445_m1) and CD44 (Hs00174139_m1). Comparative gene expression was performed using the delta-delta Ct method.

**Statistical analysis.** All error bars are represented as the standard error of the mean. Significance levels for comparison between groups were analyzed using unpaired Student’s t-test. All statistical tests were two sided and the differences were considered significant when $P$-value was $< 0.05$.

**Results**

**In vivo antitumor effects of tigatuzumab alone or in combination with GEM.** When tested as a single agent, tigatuzumab showed variable efficacy in PDA xenografts. Tigatuzumab monotherapy showed comparable antitumor activity to that of GEM in 2/8 xenografts. T/C for Panc219 and Panc286 were 17.7% and 33.9% for tigatuzumab and 44.7% and 46.3% for GEM, respectively (Fig 1A). Tigatuzumab monotherapy was not effective to achieve tumor regression even in the most sensitive xenografts (Panc219 and Panc286). Rather, the established tumors showed decreased growth compared with...
controls. GEM monotherapy showed greater efficacy resulting in tumor regression in 4/8 xenografts (Fig 1A). The combined treatment of GEM with tigatuzumab suppressed the tumor growth in 7/8 xenografts and resulted in tumor regressions in 5/8 xenografts (Fig 1A).

**Tigatuzumab triggers apoptosis through the cell-extrinsic death pathway.** Western blot analysis of tumor lysate from Panc219 xenograft showed that tigatuzumab induces DR5 expression. DR5 levels were elevated in tigatuzumab and combination treatment groups compared to control and GEM treated animals (Fig 1B). There was an average of 2.5 and 3.0-fold increase in DR-5 expression in the tigatuzumab and GEM plus tigatuzumab treated tumors, respectively, as compared to the saline treated tumors. The activation of DR5 in the tigatuzumab and combination therapy group was coupled with the up-regulation of Fas, FADD, and TRADD (Fig 1B). Tigatuzumab and GEM plus tigatuzumab treatments lead to the up-regulation of Fas (1.8 and 2.4-fold), FADD (1.5 and 2.6-fold) and TRADD (2.2 and 2.1-fold), respectively, as compared to the control tumors. GEM treatment did not modulate the expression of the above mentioned proteins (Fig 1B). There was a 1.9 and 2.4-fold decrease in the expression of full length and cleaved-caspase 8, respectively, in the GEM treated tumors as compared to the vehicle treated tumors. A similar decrease in TRAIL levels (1.8-fold) was noticed in GEM treated tumors as compared to control tumors. Both agents alone and in combination were marginally effective in reducing the XIAP expression in tumors as compared to the control tumors (Fig 1B). There was a 2.4-fold up-regulation of cleaved-PARP, a marker of apoptosis in the GEM plus tigatuzumab treated tumors as compared to the GEM treated tumors (Fig 1B). Caspase-3, BID, BAX and p-53 expression were unchanged in various treatment groups as compared to the control tumors (Fig 1B).

**Combination of GEM with tigatuzumab leads to cooperative tumor growth inhibition and prolong the tumor doubling time.** GEM treatment resulted in rapid tumor shrinkage in PDA xenografts (Fig 1A). However, tumor growth resumed within 2-4 weeks after the cessation of treatment, indicating that GEM treatment, does not result in
long-term cures (Fig 2A and B) as observed in the clinic. There was no significant difference in the mean initial tumor doubling time of vehicle and tigatuzumab treated animals (13±3 and 17±3 days, respectively). The mean initial tumor doubling time of tumors in GEM treatment was 54±26 days. However, the mean initial tumor doubling time in the combination therapy group was 92±36 days. Aggregate analysis of the initial tumor doubling time of all tumors in the eight xenografts showed a significant increase in tumor doubling time in the combination treatment group as compared to the GEM group (Fig 2C). In addition, tumors in the Panc219, Panc410, Panc374 and Panc281 xenografts of the combination group did not recur up to 120 days of follow-up (Fig 2B). There was a complete remission of some tumors in Panc219 (2/8 on day 35), Panc410 (1/8 on day 45) and Panc374 (1/7 on day 31). Histological examination of tumor injection sites of mice (on day 120), in which complete tumor remission occurred, failed to detect tumor cells (data not shown), indicating that combination therapy resulted in complete pathologic responses in these animals. The average tumor volume of Panc281 upon sacrifice on day 120 was 631mm$^3$ and 183mm$^3$ for GEM alone and GEM plus tigatuzumab group, respectively (Fig 2B). This suggests that the combination therapy is very efficacious in preventing tumor recurrence and to achieve tumor remission.

**Cancer stem cell population varies from xenografts to xenografts.** CSCs are currently identified by the expression of cell surface and functional markers. Several studies have reported that CSCs from primary human PDA can be identified based on of the expression of specific cell surface antigens including CD44 and CD24 (5, 13). Due to the molecular heterogeneity among CSCs, a single marker is unlikely to identify all CSC even within tumors of the same clinical grade from the same organ (31). Here we used the expression of ALDH$^+$ and CD24$^+$CD44$^+$, which are reliable marker of CSCs (5, 32). ALDH$^+$, CD24$^+$CD44$^+$ as well as ALDH$^+$CD24$^+$CD44$^+$ tumor cells (1000 cells) has been demonstrated to initiate tumors on injection into the NOD/SCID mouse, where as tumor did not develop in the mouse injected with 10,000 bulk tumor cells (30). These three markers were widely used to identify putative CSCs in most common cancers and in the NCI60 panel of tumor cell lines (33). When examined individually, putative stem cell
markers were heterogeneously expressed across the xenografts. There was a marked variation in CD24⁺CD44⁺ tumor cells (0.1-10.8%) among the xenografts (Table 1). Similarly, wide variations in ALDH⁺ cells (1.4-9.3 %) were also noticed among the xenografts (Table 1). We have recently reported that, the ALDH⁺CD24⁺CD44⁺ population were more tumorigenic than both the ALDH⁺ and CD24⁺CD44⁺ tumor cells, but the differences were not statistically significant (30). In addition to analyzing the CD24⁺CD44⁺ and ALDH⁺ tumor cell populations, we also analyzed the ALDH⁺CD24⁺CD44⁺ population in pancreatic cancer xenografts. Similar to the findings of our recent work (30), we found that percentage of ALDH⁺CD24⁺CD44⁺ tumor cells were too low to be accurately measured (<0.015%) and these populations were largely non-overlapping with CD24⁺CD44⁺ and ALDH⁺ tumor cells (data not shown). The four xenografts (Panc219, Panc410, Panc374 and Panc281), which did not recur up to 120⁺ days of follow-up in the combination therapy has relatively higher percentage of cancer stem cell population as compared to other xenografts (Table 1).

**DR5 levels are enriched in PDA stem cells.** We analyzed the DR5 expression in pancreatic CSCs and non-stem cell bulk tumor cells by Flow cytometry. Pancreatic CSCs are indeed enriched for DR5 expression (94% of ALDH⁺ tumor cells and 89% of CD24⁺CD44⁺ tumor cells) as compared to non-stem cell bulk tumor cells (30%) in Panc219 (Fig 3). A similar enrichment of DR5 in ALDH⁺ and CD24⁺CD44⁺ tumor cells were noticed in other xenografts used in this study (data not shown).

**Combination therapy reduces cancer stem cell populations.** Flow cytometry of tumor cells sorted from Panc219 showed that GEM treatment was not capable of diminishing the CSC pool as compared to the control mice (Fig 4). However, combination of GEM with tigatuzumab resulted in a 14.3- fold decrease in CD24⁺CD44⁺ tumor cells compared to GEM treatment (Fig 4). Similarly, there was a 2.31-fold decrease in ALDH⁺ tumor cells in the combination therapy as compared to GEM treatment (Fig 4). Tigatuzumab monotherapy reduced the cancer stem populations to nearly half to that of GEM and control mice (Fig 4).
CD24 staining index indicate that GEM treatment did not reduce CD24⁺ tumor cells as compared to control tumors (Fig 5A). However, tigatuzumab and the combination of GEM with tigatuzumab reduced the CD24 staining index in tumor cells as compared to the control and GEM treated groups (Fig 5A and B). Combination treatment resulted in the complete elimination of CD24⁺ tumor cells in Panc410 and Panc374 (Figure 5B).

Quantitative real-time reverse transcription-polymerase chain reaction results of Panc219 and Panc410 clearly showed that combination of GEM with tigatuzumab down-regulate the mRNA expression of cancer stem cell markers as compared to GEM treated tumors. GEM treatment resulted in the up-regulation of ALDH and CD44 mRNA expression as compared to control tumors (Fig 5C). However, combination of GEM with tigatuzumab reduced the elevated ALDH and CD44 levels as compared to GEM treatment (Fig 5C). There were a 4.2, 4.0 and 3.6-fold decrease in the expression of ALDH, CD24 and CD44 mRNA expression, respectively, in combination therapy as compared to GEM treatment (Fig 5C). Similarly, combination therapy resulted in a 5.3, 2.3 and 3.3-fold decrease in the expression of ALDH, CD24 and CD44 mRNA expression, respectively, in Panc410 as compared to GEM treated tumors (Fig 5C). Tigatuzumab monotherapy did not alter the ALDH, CD24 and CD44 mRNA expression as compared to vehicle treated tumors (Data not shown).

**Discussion**

Despite rapid advances in many fronts, PDA remains one of the most difficult human malignancies to treat, indicating the need for novel therapeutic strategies in order to improve the poor prognosis of patients with this disease. The existence of CSCs might probably influence the intractable nature of PDA, explaining why conventional cancer therapy fails in the vast majority of patients. CSCs have been shown to be highly resistant to chemotherapy and radiotherapy. Thus from a clinical standpoint, targeting cancer-
sustaining pancreatic CSCs will be of paramount significance since there are few effective therapies for PDA and most of the patients die within the first year of diagnosis.

In this report, we provide evidence that pancreatic CSCs are relatively enriched with DR5 compared to the non-stem cell bulk tumor populations. A combination therapy using DR5 agonist mAb, tigatuzumab and GEM produced robust antitumor activity in freshly generated PDA xenografts. More importantly, the combination therapy resulted in the reduction of pancreatic CSCs, tumor remission, prevents tumor recurrence and significantly prolongs the time to tumor progression. The combination therapy tested here has a wide potential therapeutic role, which may possibly kill cancer-sustaining stem cell pool to prevent tumor recurrence and as an adjuvant therapy in clearing cancer stem cell populations following primary tumor resection and radiation therapy.

DR5 agonists represent a new class of therapeutics that selectively target apoptosis. Significant effort has been devoted to the discovery and development of mAbs against DR5, based on the impressive tumor growth inhibition and superior survival advantage obtained in a broad range of human tumor xenografts treated with DR5 agonist mAbs in combination with chemotherapy/or radiation therapy (34-37). Activation of DR5 pathway triggers programmed cell death through extrinsic apoptotic pathway, independent of p53, which involves the direct recruitment and activation of caspase-8 through Fas-associated death domain (FADD) (38). The extrinsic apoptosis pathway is an intriguing target for cancer therapy because it can circumvent a common apoptosis resistance mechanism associated with mutations in p53 tumor suppressor gene that is accounted for 50% of human cancers (39, 40). On the other hand, many conventional chemotherapeutic agents generally require the function of p53 to initiate apoptosis by engaging the intrinsic apoptotic pathway (41). Combinations of the two approaches may facilitate tumor cell death that resist death induction through either one of the pathways as well as reduce the probability of tumor cell to develop resistance to either therapy.
Mounting data over the recent years have indicated the robust preclinical activity of TRAIL receptor targeted human agonistic mAbs across a broad range of tumor types. Based on this, multiple clinical trials either single agent or in combination with chemotherapeutics, are currently at various stages (42). Early-phase clinical trials using agonistic anti-TRAIL receptor antibodies indicate that these agents can be delivered safely, are generally well-tolerated and seem to mediate some clinical benefit in terms of disease stabilization or objective responses (43). Death receptor agonists were safely combined with standard doses of cancer chemotherapeutics in small cohorts of patients. These combinations include single-agent cytotoxics, cytotoxic combinations, targeted agents and cytotoxic-targeted agent combinations (25). Our results clearly demonstrate that GEM treatment initially results in rapid tumor shrinkage in human primary PDA xenografts. GEM treatment did not deplete the CSC population and the tumors progressed after an interim in all xenografts. However, tumors in 50% (4/8) of the xenografts treated with GEM and tigatuzumab not only did not relapse, but also did not double its size till sacrificed on day 120. Specifically, this notable co-operativity between tigatuzumab and GEM in tumor growth inhibition as well as CSC reduction suggests that this therapeutically attractive combination therapy could be efficacious in clinical setting.

Notwithstanding our ability to sequence cancer genome and to create personalized targeted therapies, it is apparent that combination therapies, which target the CSC subpopulation as well as the bulk of the tumor cells, will be required to effectively manage cancer treatment (44, 45). Emerging studies show that CSCs are indeed more resistant to therapy than other cancer cells and injection of a small number of CSCs are able to reproduce an entire tumor (46). It is thought that CSCs are relatively drug- and radiation resistant by virtue of quiescence, expression of drug resistance mechanisms, and possibly their location in tissue niches with restricted drug access. Therefore, development of novel therapeutic strategy to combat CSCs is an attractive strategy for more effective cancer treatment. Although, the true relevance of the CSC is yet to be revealed, there are tantalizing reports that the CSC can be selectively targeted without
ablating normal stem cells (47). As the concept of CSC is becoming scientifically accepted, there is increasing interest in evaluating potential therapeutic targets in these cells. Initial studies have focused on the evaluation of developmental pathways such as the Wnt and the Sonic Hedgehog pathways (48). Indeed, a recent report from our group showed that the Sonic HH inhibitor, cyclopamine, induce tumor regression in combination with GEM in one patient PDA xenograft (16). Another study showed that a combination of cyclopamine, rapamycin and GEM was capable of eliminating pancreatic CSCs (49). The present results indicate that there may be other targets in CSCs and target-focused as well as systematic approaches are needed to investigate this aspect. As novel CSC directed therapies emerge, studies such as the one presented here can be of great value when trying to prioritize drugs with anti-CSC activity for preclinical testing and validation. This may allow us to better select targeted agents directed to CSCs, which may ultimately improve the chances that the chosen therapy successfully completes the clinical development.

The quest for potent targeted therapeutic approaches to combat CSCs is an imperative clinical issue. A recent report from our group suggest that pancreatic CSCs play a key role in the development of metastatic disease that negatively affects the overall survival of patients with PDA (30). An important question is how we move forward and translate these findings to the clinic. By the time tumors are diagnosed, the disease is usually advanced and does not respond to treatment. Adding CSC directed therapeutics to conventional agents in this advanced stage may unlikely be effective. A more appropriate scenario can be patients with resectable disease, who despite treatment with GEM uniformly develop disease progression. Here, the most important clinical goal is the elimination of micrometastatic disease, in which CSCs probably have a critical role. Combination of CSC directed treatment with chemotherapy can be tested with the primary objectives of preventing disease progression, relapse and metastasis. Importantly, agents that eliminate the CSCs within a tumor may bring little or no immediate reduction in tumor size. However, tumor growth is not sustainable without CSCs to replenish the
bulk population, and the tumor will eventually degenerate as bulk tumor cells are depleted.

Our results agree with the previous report (16) that ALDH and CD44 mRNA levels were elevated in the GEM treated tumors as compared to the saline treated tumors (Fig. 5B). However, increased average mRNA levels did not correspond to an increase in the number of ALDH+ and CD24+CD44+ tumor cells as revealed by FACS data (Fig 4). At present, we do not know the reason behind this discrepancy. Additional studies are needed to investigate whether post-transcriptional modification or trafficking of antigens between cell membrane/cytoplasm and nucleus play a role on this aspect. There is now abundant evidence that stem-cell properties are highly relevant to the biology of several human cancers. An obvious question is whether cancer stem cells in other tumor types have DR5 expression? Studies are needed to investigate whether DR5 is also a CSC target in other tumor types besides pancreas cancer.

Taken together, our results provide strong evidence that pancreatic CSCs are enriched with DR5. Combination therapy using GEM plus tigatuzumab results in long-term cures in otherwise incurable direct PDA model. Furthermore, the novel combination prove to be more efficacious than either single agent alone by providing a double hit to kill both CSCs and non-stem cell bulk tumor populations. Considering that combination therapy produced remarkable reduction in PDA stem cells, tumor remission and significant improvements in time to tumor progression, this innovative approach may represent a valuable treatment option to improve the current standard of care for PDA patients. Further investigation of this promising approach in PDA is warranted.
References


**Figure legends**

**Figure 1.** Combination of GEM with tigatuzumab produce sustained tumor growth inhibition and induce the expression of cell-extrinsic apoptotic pathway proteins in pancreatic cancer xenografts. **Figure 1A.** *In vivo* efficacy of Tigatuzumab, GEM and combination of GEM with tigatuzumab on the tumor growth of established pancreatic adenocarcinoma xenografts. Tigatuzumab monotherapy was in effective in controlling the tumor growth of xenografts. However, combination of GEM with tigatuzumab was highly effective in preventing the tumor growth of xenografts. Eight individual patient-derived low passage pancreatic cancer xenografts were implanted in athymic nude mice.
Cohorts of mice with a tumor volume of 200 mm$^3$ were randomized and treated with (a) Saline (vehicle); (b) tigatuzumab (3 mg/kg, i.v, once week for four weeks); (c) GEM (100 mg/kg i.p, twice week for four weeks) and (d) GEM + tigatuzumab in the above mentioned dose and frequency. Relative tumor growth rate on day 28 of treated animals were calculated versus the tumor volume of vehicle treated mice (100%). Cases that showed maximum sensitivity to tigatuzumab were plotted on the left side of graph. Error bars represent standard error of mean (SEM); N = 10 tumors per group (4 mice with bilateral flank tumors and 2 mice with unilateral flank tumor). **Figure 1B.** Panc219 western blot showing that tigatuzumab or combination with GEM induces the expression of key proteins of extrinsic apoptotic pathway. Tumors harvested on day 28 were used for immunoblotting. Equal amounts of tumor lysates (30 μg) from two separate animals in each group were analyzed by immunoblotting and probed with the indicated antibodies. Band intensities were measured by densitometry and normalized with respective b-actin loading controls. There were an average of 2.5 and 3.0-fold increase in DR-5 expression in the tigatuzumab and GEM plus tigatuzumab treated tumors, respectively, as compared to the saline treated tumors. The activation of DR5 in the tigatuzumab and combination therapy group was coupled with the up-regulation of Fas, FADD, and TRADD. Tigatuzumab and GEM plus tigatuzumab treatments lead to the up-regulation of Fas (1.8 and 2.4-fold), FADD (1.5 and 2.6-fold) and TRADD (2.2 and 2.1-fold), respectively, as compared to the control tumors. GEM treatment did not modulate the expression of the above mentioned proteins. A 1.9 and 2.4-fold decrease in the expression of full length and cleaved-caspase 8 in the GEM treated tumors as compared vehicle treated tumors. A similar down-regulation of TRAIL (1.8-fold) was noticed in GEM treated tumors as compared to the vehicle treated tumors. Both agents alone and in combination were marginally effective in reducing the XIAP expression in tumors as compared to the control tumors. There was a 2.4-fold up-regulation of cleaved-PARP, a marker of apoptosis in the GEM plus tigatuzumab treated tumors as compared to the GEM treated tumors.
Figure 2. Combination therapy produces durable tumor growth inhibition and prolongs the tumor doubling time of pancreatic cancer xenografts. Figure 2A. Initial tumor doubling time of eight xenografts treated with tigatuzumab, GEM, and combination of GEM with tigatuzumab. Tumors in the combination treatment group of Panc219, Panc410, Panc374 and Panc281 did not double its size as on day 120 compared to the initial tumor size. Asterisk mark indicates that tumors in that group did not double its size upon necropsy. There were 10 tumors each in various treatment groups till day 28 and thereafter 6-8 tumors in the GEM and GEM plus tigatuzumab group in each xenografts. Error bars represent the standard error of mean (SEM). Figure 2B. Combination of GEM with tigatuzumab produce durable tumor growth inhibition in pancreatic cancer xenografts. Tumor growth curves representative Panc219, Panc410 and Panc374. Numbers near to asterisk mark denotes the number of tumors vanished during treatment. There were 10 tumors each in various treatment groups till day 28 and thereafter 6-8 tumors in the GEM and GEM plus tigatuzumab group in each xenografts. Representative images of live, anaesthetized mice treated with GEM and GEM plus tigatuzumab of Panc281; excised tumors on day 120 are showed on the lower right end of the figure. Figure 2C. Log-rank comparison of aggregate initial tumor doubling time of GEM (N=53 and GEM plus tigatuzumab (N=58) treated tumors of eight xenografts. Animals in the combination treatment resulted significant increase in time to tumor doubling compared to GEM treatment (p=0.002).

Figure 3. DR5 is enriched in PDA stem cells as compared to the bulk tumor cells. Tumors from mice bearing Panc219 was harvested and single cell suspensions were generated by mincing tumors using sterile razors, followed by incubation in dispase and collagenase type IV at 37°C for 2h with agitation. DR5⁺ tumor cells in the bulk tumor populations and in ALDH⁺ and CD44⁺CD24⁺ tumor cells were measured by FACS as described in the materials and methods. The frames represent the gates that depict respective antigen positive tumor cells. DR5 is expressed in only 30% of bulk tumor cells. However, CSCs are relatively enriched with DR5 expression (94% of ALDH⁺ cells and 89% of CD24⁺CD44⁺ cells) as compared to the bulk tumor cells.
**Figure 4.** Combination of GEM with tigatuzumab markedly enhances the elimination of PDA stem cells. Panc219 tumor bearing mice were treated with tigatuzumab, GEM and GEM plus tigatuzumab as mentioned in the materials and method. The tumors were harvested on day 28 and single cell suspension was generated and cells positive for cancer stem cells markers were measured using Flow cytometry. The frames represent the gates that depict respective antigen positive tumor cells. Remarkable decrease in CD24^+CD44^+ and ALDH^+ tumor cells (14.3 and 2.31-fold, respectively) were noticed in animals that received combination therapy as compared to GEM treatment.

**Figure 5.** Combination therapy reduces PDA stem cells as demonstrated by immunohistochemical staining and qRT-PCR. **Figure 5A.** CD24 staining index of Panc219, Panc410 and Panc374. Immunohistochemical staining of CD24 positive tumor cells were performed as described in the materials and method section. Index means % of positively stained tumor cells X staining intensity (0, 1, 2, and 3). GEM treatment was not capable of reducing the CD24^+ tumor cells as compared to vehicle treated tumors. **Figure 5B.** Representative micrographs of CD24 immunohistochemical staining of Panc219, Panc410 and Panc374 xenografts showing low immunoreactivity for CD24 in tigatuzumab and combination of GEM with tigatuzumab group as compared to vehicle treated and GEM treated tumors. Combination therapy leads to the complete elimination of CD24 stained tumor cells in Panc410 and Panc374 xenograft tumors. **Figure 5C.** Combination of GEM with tigatuzumab down-regulate the mRNA expression of cancer stem cell markers as compared to GEM treated tumors. RNA isolated from Panc219 and Panc410 treatment groups were used for qRT-PCR and comparative mRNA expression of ALDH, CD44 and CD44 were calculated using the delta-delta CT method. In Panc219, there were a 6 and 3.3-fold up-regulation of ALDH and CD44 mRNA in GEM treated tumors, respectively, as compared to the control tumors. However, the combination therapy leads to a 4.2, 4.0 and 3.6-fold decreases in the expression of ALDH, CD24 and CD44 mRNA expression, respectively, as compared to GEM treatment (Fig 5C). Similarly, there were a 8.4 and 2.3-fold increase in the mRNA expression of ALDH and...
CD44, respectively, in the GEM treated tumors of Panc410 as compared to the control tumors. Combination therapy resulted in 5.3, 2.3 and 3.3-fold decrease in the expression of ALDH, CD24 and CD44 mRNA expression, respectively, in Panc410 as compared to GEM treated tumors (Fig 5C).
Fig 1.

A

B

Patient Pancreatic Tumors Xenografted in Mice

TRAIL

BID

C-Casp-3

Fas

C-Casp-8

FADD

Casp-8

TRADD

Casp-3

DR5

BAX

p57/43

C-PARP

FADD

p50/40

β-Actin

p32

p53

TRADD

p28

p18

TRAIL

p34

p20

p19/17

p15

p20

p89

p53

p53

p45
Fig 2.

A) Xenografts

- Horizontal axis: days from 0 to 120
- Vertical axis: tumor doubling time (in days)
- Bars represent different treatments:
  - Control
  - Tigatuzumab
  - GEM
  - GEM + Tigatuzumab

B) Percentage tumor growth (Mean)

- Graph shows percentage tumor growth over time for different treatments:
  - Panc219
  - Panc281
  - Panc410
  - Panc374

C) Doubling of initial tumor volume

- Graph shows the doubling of initial tumor volume over time for GEM and GEM + Tigatuzumab treatments.
  - GEM (n=53)
  - GEM + Tigatuzumab (n=58)
  - p-value: 0.002

Average tumor volumes:
- Panc281 GEM: 631 mm³ (on day 120)
- Panc281 GEM + Tigatuzumab: 183 mm³ (on day 120)
Fig 3.

Bulk Population

CD44

CD24

SSC

ALDEFLUOR (ALDH)

Cell #

DR5

5.1%

4.62%

30.1%

CD24^+CD44^+ Population

ALDH^+ Population

Cell #

DR5

89.4%

94.3%
Table 1: Stem cell quantification in xenografts by Fluorescence Activated Cell Sorting (FACS)

<table>
<thead>
<tr>
<th>Xenografts</th>
<th>% ALDH+</th>
<th>% CD44+CD24+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panc219</td>
<td>5.30</td>
<td>6.20</td>
</tr>
<tr>
<td>Panc281</td>
<td>9.05</td>
<td>3.13</td>
</tr>
<tr>
<td>Panc185</td>
<td>1.40</td>
<td>0.10</td>
</tr>
<tr>
<td>Panc198</td>
<td>4.70</td>
<td>10.10</td>
</tr>
<tr>
<td>Panc374</td>
<td>4.40</td>
<td>9.30</td>
</tr>
<tr>
<td>Panc410</td>
<td>9.30</td>
<td>0.34</td>
</tr>
<tr>
<td>Panc253</td>
<td>2.30</td>
<td>0.10</td>
</tr>
<tr>
<td>Panc286</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Note: Eight patient-derived pancreatic tumors grown separately (s.c) in nude mice were harvested. Single cell suspensions were generated by mincing tumors using sterile razors, followed by incubation in Dispase (Sigma) and Collagenase IV (Sigma) at 37°C for 2h. The cell suspension was filtered using a 70-micrometer filter (BD Biosciences) and further purified by density centrifugation using Ficoll-Paque Plus (GE Healthcare). Cells were labeled using the ALDEFLUOR reagent (Stem Cell Technologies) and then stained with antibodies against mouse CD31 (BD Biosciences), lineage cocktail (Miltenyi Biotec), and H-2Kd (BD Biosciences). Non-mouse cells were separated and stained with antibodies against human CD44, CD24 (BD Biosciences). Cells were analyzed using a FACS Aria Flow Cytometer (BD Biosciences). Due to the abundance of necrosis in Panc286, we did not have enough cells to sort this case for stem cell assessment.
Molecular Cancer Therapeutics

A combination of DR5 agonistic mAb with gemcitabine targets pancreatic cancer stem cells, and results in long-term disease control in human pancreatic cancer model

N.V. Rajeshkumar, Zeshaan A Rasheed, Elena García-García, et al.

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