

## ORIGINAL ARTICLE

# Genexol inhibits primary tumour growth and metastases in gemcitabine-resistant pancreatic ductal adenocarcinoma

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## Abstract

**Introduction:** Gemcitabine, the current standard of care for pancreatic ductal adenocarcinoma (PDA), has a less than 10% partial response rate. Genexol-PM, a modified form of paclitaxel, has been shown to have antitumour effects in clinical trials of metastatic breast and small-lung-cell carcinoma. The aim of the present study was to determine if Genexol would be a beneficial treatment for gemcitabine-resistant PDA.

**Materials and methods:** We measured the *in vitro* IC50s of gemcitabine and genexol in cell lines sensitive and resistant to gemcitabine. *In vivo*, animals with orthotopic pancreatic tumours, resistant to gemcitabine, were treated with phosphate-buffered saline (PBS), gemcitabine, Genexol or gemcitabine+Genexol. Tumour progression was monitored using red fluorescent protein imaging.

**Results:** We showed equivalent IC50s for gemcitabine-sensitive and gemcitabine-resistant cell lines when treated with genexol. *In vivo* treatment with genexol resulted in a greater per cent reduction in tumour size, less metastatic spread and longer survival compared with treatment with gemcitabine.

**Discussion:** Genexol proved to be an effective treatment for gemcitabine-resistant PDA. These data combined with the successful clinical use of genexol in Phase II trials of other malignancies suggests it maybe an effective treatment for pancreatic cancer, specifically for those patients resistant to gemcitabine.

## Keywords

chemotherapy, metastases, pancreatic cancer

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## Introduction

Pancreatic ductal adenocarcinoma (PDA) is an exceptionally lethal disease, with approximately 80% of patients presenting with unresectable disease as a result of metastases and local invasion.<sup>1</sup> While survival in many cancers, including breast and colon, have increased as the result of advances in solid tumour therapies, it has been estimated that the overall 5-year survival rate for patients with pancreatic cancer is only 5%.<sup>2</sup>

The main line of defence against pancreatic cancer is gemcitabine, a nucleoside analogue, which stops tumour growth by blocking DNA synthesis. Although gemcitabine is a relatively well-tolerated drug, acquired as well as an initial non-response in PDA patients is a major limitation of this line of therapy.<sup>3</sup> The median survival with advanced disease remains less than 6 months, the overall tumour response rate is less than 25% and mean survival benefit when given post-surgical resection is 6–8 months.<sup>3,4</sup> Taken together the data suggests that a novel treatment that acts through a different pathway would offer advantages, as it could potentially work on those individuals that do not respond to gemcitabine, as well as those that acquire a resistance to gemcitabine.

One potential novel drug is Genexol. Genexol is a modified form of paclitaxel, which does not have the same toxicity problems

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that are encountered when treating with paclitaxel. The mechanism of action of Genexol at the cellular level is the stabilization of microtubules, thus preventing cell division. To date clinical trials with Genexol for the treatment of advanced malignancies, advanced small-cell-lung cancer and metastatic breast cancer have been promising.<sup>5-7</sup>

In terms of pancreatic cancer, there is preliminary data *in vitro* and in an animal model that suggests Genexol could be advantageous as a treatment regimen for pancreatic cancer.<sup>8</sup> The results of a Phase II trial for the treatment of locally invasive and metastatic PDA patients was recently published, with a median overall survival of 6.5 months.<sup>9</sup> Therefore, the aim of the present study is to examine Genexol as a potential treatment for gemcitabine-resistant pancreatic cancer *in vitro* and with an orthotopic animal model.

## Materials and methods

### Cell culture

The human pancreatic cancer cell line MiaPaCa-2 was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). Cells were cultured at 37°C in a 5% CO<sub>2</sub> incubator. The MiaPaCa-2 cells were engineered to stably express red fluorescent protein (Clontech, Palo Alto, CA), as previously described.<sup>10,11</sup> After stable transfection of red fluorescent protein (RFP), gemcitabine-resistant cells (GEM-MiaPaCa-2) were generated.<sup>12-14</sup> We have consistently shown that repeated passages of our MiaPaCa-2 cells results in a gemcitabine-resistant cell line, without exposure to gemcitabine.<sup>15</sup> We initially conducted an *in vitro* study to demonstrate that our cell line is resistant to gemcitabine.

### Drug sensitivity

The two cell lines, GEM-MiaPaCa-2 and MiaPaCa-2, were subsequently exposed to serial dilutions of gemcitabine, paclitaxel, and Genexol to determine their respective IC<sub>50</sub>s. Specifically, 500 000 cell/well were plated onto a 96-well plate, allowed to adhere for 24 h and then exposed to fresh drug and media every day for 72 h. The dosages of gemcitabine were 0, 3.9 nM, 7.8 nM, 15.6 nM, 31.25 nM, 62.5 nM, 125 nM, 250 nM, 500 nM and 1000 nM. The serial dosages for paclitaxel (Sigma-Aldrich, St. Louis, MO, USA) and Genexol (Samyang Corp, Daejeon, South Korea) were 0, 0.625 nM, 1.25 nM, 2.5 nM, 5 nM, 10 nM, 20 nM, 40 nM and 80 nM. At 72 h cells were incubated in a 10% alamar-Blue solution (Invitrogen, Carlsbad, CA, USA) for 2 h at 37°C, and then fluorescence read (FLx800; BioTek, Winooski, VT, USA) according to the manufacturer's instructions. IC<sub>50</sub>s were then determined using commercially available software (CalcuSyn, Cambridge, UK). All experiments were done in triplicate.

### Surgical procedures

Thirty male nude mice between 4 and 6 weeks of age were maintained in a barrier facility equipped with HEPA-filtered racks. All

studies were conducted with the approval and guidance of the University of Utah Institutional Animal Care and Use Committee. The orthotopic tumour induction surgery was performed as previously described.<sup>10,11</sup> Briefly,  $1.5 \times 10^6$  RFP expressing GEM-MiaPaCa-2 cells suspended in 150  $\mu$ l of serum-free media were injected into the tail of the pancreas via a left subcostal incision. The abdomen was closed using 6-0 silk sutures closing both skin and muscle simultaneously. All procedures were done under aseptic conditions.

### Imaging

After the primary surgery, high resolution (3456  $\times$  2304 pixels) whole body digital images (EOS Digital Rebel, Canon USA, Lake Success, NY, USA) of each mouse were obtained once a week to monitor primary tumour growth. We have previously showed that the RFP tumour area is linearly correlated with tumour mass.<sup>10,11</sup> The RFP was visualized with an Illumatool Bright Light System that consisted of a 563-nm excitation filter and a 587-nm viewing filter (Model LT-9900, LightTools Research, Encinitas, CA, USA). Animals were imaged under nose-cone induced isoflurane general anaesthesia. Primary tumour area was quantified using public domain software (National Institutes of Health Image; <http://rsb.info.nih.gov/ij/>).

### Treatment

At 4 weeks post-tumour induction 27 of the 30 animals had confirmed tumours, as determined by RFP imaging. Animals with tumours were randomly assigned to one of four groups: Control (Saline), Gemcitabine (3.5 mg/200  $\mu$ l/animal), Genexol (0.5 mg/200  $\mu$ l/animal) and Gemcitabine+Genexol (3.5 mg/200  $\mu$ l/animal and 0.5 mg/200  $\mu$ l/animal, respectively). The animals were treated twice a week for 2 weeks, followed by 2 weeks of no treatment, and then subsequently retreated twice a week for 2 weeks (Fig. 1). Tumour size was monitored weekly and when the tumour reached roughly 2 cm in diameter the animal was necropsied.

At necropsy, after *in vivo* imaging, the primary tumour was removed and weighed. Additionally, all metastases were located and sites of metastases noted, as well as a sample removed. All removed tumours and metastases were fixed in 10% formalin, embedded in paraffin and stained for histological analysis. RFP imaging was utilized to confirm the presence and location of any metastasis.

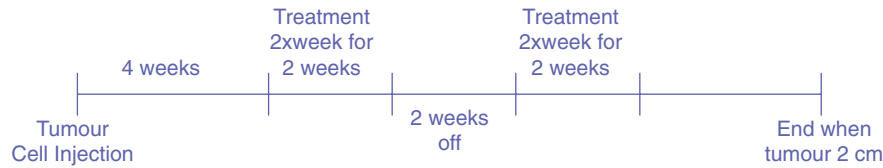
### Statistics

Group differences for final tumour mass, survival, number of metastatic sites and percent reduction in tumour size were compared utilizing an ANOVA followed by Tukey's post-hoc test. Weekly tumour size as determined by RFP imaging was compared between the groups with a repeated measure ANOVA followed by a post-hoc test. A *P*-value of <0.05 was considered significant.

## Results

### *In vitro* IC<sub>50</sub>s

As expected the IC<sub>50</sub> for GEM-MiaPaCa-2 (331  $\pm$  42 nM) was considerably higher, 10-fold, than our original wild-type



**Figure 1** Experimental timeline. Animals were treated twice, both for two times a week for 2 weeks. The endpoint was defined as when the tumour reached 2 cm in diameter as measured with red fluorescent protein (RFP) imaging

**Table 1** *In vitro* IC50s\* for three different drug formulations

Cell lines	Gemcitabine	Taxol	Genexol
MiaPaCa2	29.6 ± 1.7	5.5 ± 0.8	13.7 ± 3.8
GEM-MiaPaCa2	330.5 ± 42.2	4.5 ± 0.8	13.1 ± 4.2

\*All dosages in nM.

MiaPaCa-2 cell lines ( $30 \pm 2$  nM) (Table 1). The IC50 for paclitaxel was significantly less than gemcitabine and Genexol, with no differences between the two cell lines. Additionally, the IC50 for Genexol was less than half the dose of gemcitabine, 13 nM compared with 30 nM. Thus, both cell lines appear to be equally sensitive to paclitaxel and Genexol and the IC50 dose for paclitaxel and Genexol is smaller than gemcitabine.

### **In vivo weekly tumour area**

There were no differences between the four groups in terms of initial tumour area as measured by RFP imaging (Table 2). However, at the end of the first 2 weeks of treatment there were marked differences in tumour RFP area between the groups (Fig. 2). Specifically, at week three both the Control ( $429 \pm 113$  mm<sup>2</sup>) and Gemcitabine ( $354 \pm 76$  mm<sup>2</sup>) groups had significantly larger tumours than the Genexol ( $44 \pm 47$  mm<sup>2</sup>) and Gemcitabine+Genexol ( $34 \pm 49$  mm<sup>2</sup>) groups. The size of the Control and Gemcitabine groups' tumours had increased  $57 \pm 17\%$  and  $57 \pm 9\%$  relative to their starting areas by week three, respectively. While the tumours in the Genexol group had shrunk by  $64 \pm 38\%$  and the Gemcitabine + Genexol had decreased by  $84 \pm 17\%$ , all of the tumours in the Control and Gemcitabine groups had reached 2 cm at 4 weeks, thus the animals in these two groups did not receive the second round of treatment. Although the Genexol-treated groups manifested significant tumour regression during the first 2 weeks of treatment, the second 2-week treatment did not cause regression of residual tumours (Fig. 2). By week 7 the tumours in both the Genexol and Gemcitabine+Genexol groups were increasing in size. There were no differences in terms of absolute, relative or per cent change in tumour area between the Genexol and Gemcitabine+Genexol groups at anytime point.

### **Survival**

There was a clear difference in the survival of the Control ( $43 \pm 5$  days) and Gemcitabine ( $46 \pm 4$  days) groups compared with the Genexol ( $107 \pm 32$  days) and Gemcitabine+Genexol groups

( $103 \pm 15$  days) (Table 2; Fig. 3). Four weeks after the initiation of treatment all of the tumours in the Control and Gemcitabine groups had reached 2 cm. The Genexol and Gemcitabine+Genexol groups had a mean survival of 15 weeks (Table 2).

### **Tumour burden**

The final tumour area as measured with RFP and the final tumour mass did not differ between the four groups. In terms of metastases, 100% of the Control and Gemcitabine animals had metastases at necropsy, compared with 71% of the Genexol and 84% of the Gemcitabine+Genexol. The most frequent sites of metastases, irrespective of group, were the mesentery, diaphragm, liver and kidney. The Control ( $3.7 \pm 1$ ) and Gemcitabine ( $3.8 \pm 0.4$ ) groups had statistically more sites of metastases compared with the Genexol ( $1.6 \pm 1.3$ ) and Gemcitabine+Genexol ( $2.2 \pm 1.5$ ) groups. Roughly 70% of the animals in the Control, Gemcitabine and Gemcitabine+Genexol groups had ascites at necropsy, whereas only 42% of the animals in the Genexol groups had ascites.

### **Discussion**

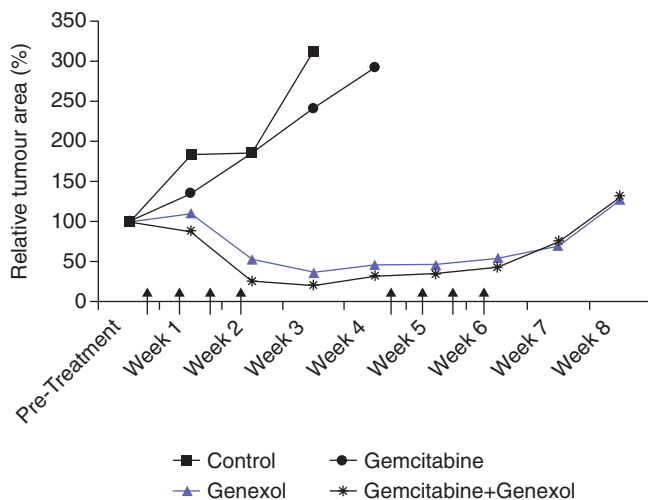
We have shown in the present study that Genexol is a potential drug to treat gemcitabine-resistant pancreatic adenocarcinoma. Initially we confirmed that our GEM-MiaPaCa-2 cells were resistant to gemcitabine, as GEM-MiaPaCa-2 cells had an IC50 that was ten times greater than our original MiaPaCa-2 cells. Next, we demonstrated equivalent IC50s for Genexol in the two cell lines. To confirm our *in vitro* data we compared gemcitabine, Genexol and the two in combination as a treatment in an orthotopic animal model of pancreatic cancer. There were dramatic differences in the response to treatment with a per cent reduction in tumour size, as measured by RFP, number of metastases and survival all showing more positive results with Genexol treatment.

These results are important, as gemcitabine resistance is a major problem in the treatment of pancreatic cancer. Less than 10% of pancreatic adenocarcinoma patients undergoing gemcitabine treatment show a partial response.<sup>4</sup> As gemcitabine is currently the gold standard of care, resistance is a dramatic problem. There are several mechanisms by which pancreatic cancer can acquire resistance to gemcitabine. Numerous studies have shown a positive correlation between hENT1, the main nuclear transporter that brings gemcitabine into the cell, and

**Table 2** *In vivo* experimental endpoints for the different treatment regimens

Group	Initial tumour area (mm <sup>2</sup> )	Final tumour area (mm <sup>2</sup> )	Final tumour mass (g)	Survival (days)	Per cent metastasize
Control	148 ± 59	367 ± 89	4.0 ± 1.4	43 ± 5*	100*
Gemcitabine	151 ± 50	364 ± 82	3.4 ± 1.2	46 ± 4*	100*
Genexol	137 ± 50	317 ± 101	3.8 ± 1.5	107 ± 32	71
Gemcitabine+Genexol	175 ± 64	367 ± 43	3.9 ± 1.1	103 ± 15	84

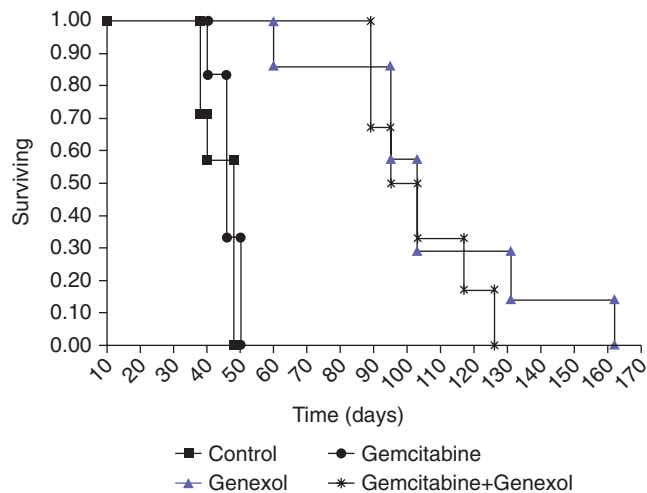
\*Statistically different from Genexol and Gemcitabine+Genexol;  $P < 0.05$ .



**Figure 2** Graph of relative tumour area by week for the four treatment groups. The Control and Gemcitabine groups had statistically larger tumours by 2 weeks after the start of treatment. The Genexol and Gemcitabine+Genexol groups had dramatic decreases in tumour area during the first set of treatments, but tumours appeared to remain the same size during the second set of treatments. Arrows indicate when the animals were treated

survival, as well as gemcitabine sensitivity.<sup>16–18</sup> Other mediators of gemcitabine resistance include deoxycytidine kinase and ribonucleotide reductase.<sup>19,20</sup> Reductions in the function of these kinases results in an increase in gemcitabine resistance, as both are important components in the metabolism of gemcitabine. Although the elucidation of the mechanism of gemcitabine resistance is beyond the scope of the present study, we show that cells resistant to gemcitabine are sensitive to Genexol, both *in vitro* and *in vivo*. Thus, Genexol maybe an important second line of defence in the treatment of pancreatic cancer, as the animals treated with Genexol survived on average three times as long as those treated with gemcitabine.

The mechanism of action of gemcitabine differs from that of Genexol and paclitaxel. Gemcitabine acts by stabilizing DNA.<sup>3</sup> In contrast, Genexol and paclitaxel stabilize microtubules.<sup>6,21</sup> Thus, Gemcitabine acts at the genetic level by preventing the replication of DNA, whereas Genexol and paclitaxel physically prevent the cells from dividing by stabilizing the microtubules. Genexol may be advantageous to use as a treatment regimen compared



**Figure 3** Survival graph for the four treatment groups. The Genexol and Gemcitabine+Genexol groups lived longer on average than the Control and Gemcitabine treatment groups

with paclitaxel. The micelle formulation of Genexol alleviates the need to use Cremophor EL, thus reducing the cytotoxicity of the drug.<sup>6</sup> Paclitaxel has poor water solubility, so Cremophor EL is required to increase the solubility of the drug to allow for systemic delivery. However, Cremophor EL is also associated with toxicity.<sup>22</sup> Genexol has a larger maximum tolerated dose than paclitaxel, probably the result of the different formulations.<sup>6</sup>

Given these benefits of Genexol there have been several clinical trials evaluating the efficacy of Genexol. In the Phase II trial for metastatic breast cancer 24 of the 41 patients (59.5%) were either complete or partial responders, with a median time to progression of 9 months. A Phase II trial for patients with advanced non-small-cell lung cancer, reported a response rate of 37.7%.<sup>5</sup> The clinical trials suggest that Genexol maybe a beneficial chemotherapy agent for the treatment of PDA, as such a Phase II trial has recently been published. Patients with metastatic or locally invasive PDA were treated with Genexol at a dose of 300 mg/m<sup>2</sup> every 3 weeks.<sup>9</sup> The disease control rate was 65%, with a median overall survival of 6.5 months. The present investigation suggests the Genexol could also be a useful treatment option for PDA patients that are insensitive to gemcitabine.

## Conclusion

Gemcitabine resistance is a major obstacle in the treatment of PDA. Genexol treatment resulted in a reduction in tumour mass, as measured by RFP, a reduction in the incidence of metastases and increased survival in a gemcitabine-resistant orthotopic animal model of PDA. Clinical trials for the treatment of metastatic breast cancer and non-small-cell lung cancer, accompanied by the data presented here suggest that Genexol could be a good second line of defence against pancreatic cancer, specifically for patients resistant to gemcitabine.

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## Conflicts of interest

None declared.

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