Original Article

1,2,3-Triazolyl ester of ketorolac (15K), a potent PAK1 blocker, inhibits both growth and metastasis of orthotopic human pancreatic cancer xenografts in mice

Rene Hennig[1], Alia Albawardi[2], Saeeda Almarzooqi[2], Shoja Haneefa[3], Edward Imbaraj[3], Nur Elena Zaaba[3], Abderrahim Nemmar[3], Sandeep Subramanya[3], Hiroshi Maruta[4,*], Thomas E. Adrian[3,5,*]

[1] *Department of General and Visceral Surgery, Freudenstadt University Hospital, Freudenstadt, Germany;*
[2] *Department of Pathology, United Arab Emirates University, Al Ain, UAE;*
[3] *Department of Physiology, United Arab Emirates University, Al Ain, UAE;*
[4] *PAK Research Center, Melbourne, Australia;*
[5] *Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, UAE.*

Summary   **More than 90% of human pancreatic cancers carry the oncogenic mutant of Ki-RAS and their growth depends on its downstream kinase PAK1, mainly because PAK1 blocks the apoptosis of cancer cells selectively. We developed a highly cell-permeable PAK1-blocker called 15K from an old pain-killer (ketorolac), that is shown here to inhibit the growth of three pancreatic cancer cell lines with IC$_{50}$ values ranging 41-88 nM *in vitro*. The anti-cancer effect of 15K was further investigated in an orthotopic xenograft model with gemcitabine (GEM)-resistant human pancreatic cancer cell lines (AsPC-1 and BxPC-3) expressing luciferase in athymic mice. During 4 weeks, 15K blocks total burden (growth) of both AsPC-1 and BxPC-3 tumors (measured as radians/sec) with the IC$_{50}$ below daily dose of 0.1 mg/kg, *i.p.* In a similar manner 15K reduced both their invasion and metastases as well, while it had no effect on either body weight or hematological parameters even at 5 mg/kg/day. To the best of our knowledge, 15K is so far the most potent among synthetic PAK1-blockers *in vivo*, and could be potentially useful for therapy of GEM-resistant cancers.**

Keywords: 1,2,3-Triazolyl ester of ketorolac (15K), PAK1, pancreatic cancer, gemcitabine-resistance, xenografts

1. Introduction

The oncogenic/ageing kinase PAK1 (RAC/CDC42-activated kinase 1) is abnormally activated by the oncogenic RAS mutants, and essential for both growth and metastasis of RAS-transformed malignant cells

(RAS cancers) such as those seen in more than 90% of pancreatic cancers, 50% of colon cancers and 30% of lung cancers in humans (*1*). Moreover, only 10% of human pancreatic cancers are sensitive to gemcitabine (GEM), and the rest is highly resistant to GEM treatment. Interestingly, a major reason for GEM-resistance is GEM-induced abnormal activation of PAK1 (*2*). Thus, potent PAK1-blockers could effectively overcome their GEM-resistance. Indeed, the combination of AG879 (inhibitor of a Tyr-kinase ETK, 20 mg/kg) and PP1 (inhibitor of another Tyr-kinase Src, 20 mg/kg) that block the activation of PAK1 strongly suppresses almost completely the growth of human pancreatic cancer xenografts in mice (*3*). However, both AG879 and PP1 are water-insoluble, and their low-bioavailability renders them clinically unfeasible. Thus, we have been developing a series of water-soluble

and highly cell permeable PAK1 blockers which are potentially useful for clinical use.

Recently, we synthesized 1,2,3-triazolyl ester of an old pain-killer (ketorolac) *via* Click Chemistry which is both highly cell-permeable and water-soluble (*4*). Ketorolac is a synthetic COOH-bearing "racemic" pain-killer that inhibits directly COX-2 in S-from (*5*). However, recently R-form of ketorolac was found to inhibit RAC/CDC42 directly, therefore blocking the down-stream kinase PAK1 (*6*). Unfortunately, due to its COOH moiety that hampers the free penetration through the negatively charged phospholipid-bilayers of target cell membranes, its cell-permeability *per se* is very poor (anti-cancer IC_{50} around 13 μM). Thus, *via* Click Chemistry, we have esterized ketorolac with the water-soluble 1,2,3-triazolyl alcohol, making 15K which is over 500 times more cell-permeable than ketorolac, and inhibits the growth of RAS-transformed lung cancer cell line (A549) with IC_{50} around 24 nM. Furthermore, 15K was found to extend the healthy lifespan of *C. elegans* 15-30% at 50 nM (depending on the temperature) and boost its heat-resistance over 9 times (*7*). Thus, it is strongly suggested that 15K, a potent anti-cancer (anti-PAK1/anti-COX-2) and anti-aging drug, could overcome the GEM-resistance of human pancreatic cancers without any serious side-effects.

In the present study we examined the effect of 15K *in vitro* and on both growth and metastasis of GEM-resistant human pancreatic cancers orthotopically grafted in athymic mice.

2. Materials and Methods

2.1. *Chemicals*

1,2,3-Triazolyl ester of ketorolac (15K) was chemically synesized from the racemic mixture of ketorolac *via* Click Chemistry as previously described in detail (*4*). A stock solution of 15K of 10 mg/mL in DMSO was made. Fresh daily dilutions were made at concentrations of 10, 100 and 500 μg/mL for the low, medium and high doses of drug for daily injection, respectively.

2.2. *Cell lines and culture*

Firefly luciferase stably expressing human pancreatic adenocarcinoma cell lines, including AsPC-1 (pLL3.7-luc transfected; AsPC-1/CMV-Luc) and BxPC-3 (pMSCV-luc transfected; BxPC-3-Luc#2) cells, were obtained from Takashi Murakami, Faculty of Medicine, Saitama Medical University 38 Moro-Hongo, Moroyama, Saitama 350-0495, Japan. AsPC-1 cells are poorly differentiated and carry a homozygous KRAS mutation in codon12: GGT(Gly)>GAT(Asp) (*8*). BxPC-3 cells have a moderate degree of differentiation (*9*), have no KRAS mutation (*8*), but have high expression

of cancer stem cell markers (*10*). Both of these cell lines are relatively resistant to gemcitabine and become more resistant following treatment with the drug (*11-13*). In previous studies IC_{50} of GEM against BxPC-3 have been reported as ~100 nM and against AsPC-1 cells ~200-500 nM (*13,14*). Parent AsPC-1 and BxPC-3 cells were purchased from the American Type Culture Collection (Manassas VA), while S2013 cells were a generous gift from Dr. Takeshi Iwamura (Miyazaki Medical College, Japan). All Human pancreatic cancer cell lines were cultured in RPMI 1640 medium (Gibco) supplemented with L-glutamine and 10% fetal bovine serum (Sigma) as described previously (*14*).

2.3. *Monitoring the cell viability of pancreatic cancer cell lines in vitro under 15K treatment*

The cells were regularly seeded into 75-cm^2 flasks with media changes every second or third day. For experiments, cells were grown to 70% confluence, digested with trypsin-EDTA, and plated in 24-well plates and incubated at 37°C for 24 h with 10% fetal calf serum, allowing cells to adhere to the substratum. After cultured in a serum-free medium for another 24 h, they were treated with 15K at different concentrations for 72 h. RAS-transformed cells such as the majority of pancreatic cancer cells need no serum growth factor for their growth *per se*, as they produce/secrete the essential autocrine growth factors that activate PAK1. At the end of each time period, the cells were trypsinized to produce a single cell suspension, and the viable cell number, determined by trypan blue exclusion, in each well was counted using an improved Neubauer chamber.

2.4. *Orthotropic xenografts of human pancreatic cancers in mice*

For cancer xenografts, 12-16 week-old athymic NMRI nude mice (nu/nu, Charles River, Suizfeld, Germany) were bred in the UAEU animal facility. Forty 40 female mice were used for the experiment with AsPC-1 cell transplants and 40 male animals for the BxPC-3 transplants.

The mice were housed in micro-isolator cages in a filtered-air laminar flow cabinet (EuroBioConcept, Paris, France), handled under aseptic conditions and fed with autoclaved laboratory rodent food pellets. The animal protocol was approved by the Institutional Animal Care and Use Committee and all procedures were conducted to conform with Institutional Guidelines that are in compliance with College of Medicine & Health Sciences, National and International Laws and Policies (EEC Council Directive 86/609, OJ L 358, 1, 12th December 1987; and NIH Guide for Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985). Animal weight was recorded weekly.

Transplants were performed as previously described in detail (*15*). For surgery, mice were anesthesized intraperitoneally with 0.05 mL of a mixture of 0.4 mL of ketamine (Fort Dodge Animal Health, Fort Dodge, IO), 0.1 mL of xylazine (Phoenix Scientific, Inc., St. Joseph, MO), and 0.5 mL of NaCl. The abdomen was sterilized with alcohol pads and a 0.5-cm midline incision was performed. The abdominal wall was wrapped with wet gauze. After pulling the stomach on the surface, the pancreas was then carefully exposed and tumor cells (5×10^5 in 10 µL of DMEM) were injected into the duodenal lobe using a Monoject 200 27-gauge x 1/2 in. polypropylene hub hypodermic needle (Kendall, Mansfield, MA) and a 50-µL sterile glass syringe (Hamilton Company, Reno, NV). Female mice received transplants of AsPC-1 cells and male mice received BxPC-3 transplants. The needle was kept in place for 30 seconds and then carefully withdrawn and the injection sealed with a dry cotton tip to prevent leakage from the injection site. After the stomach and pancreas were returned to the peritoneal cavity, the incision was closed in two layers with vicryl-coated Rapide sutures 4-0 (Ethicon, Inc., Somerville, NJ). The animals were kept under a heating lamp and once they were ambulatory, they were returned to the laminar flow cabinet. The mice were kept in a sterile environment throughout the procedure.

2.5. *15K therapy in vivo*

One day after surgery, the male and female mice were each randomized into four groups of ten mice: Four days after transplant treatment began. Group I (Control) received daily *i.p.* injection of 0.9% NaCl solution at 10 µL/gram body weight. Groups 2, 3 and 4 received 15K at doses of 100 µg, 1 mg, and 5 mg/kg/day, respectively (10 µL/gram of each of the fresh dilutions mentioned above).

2.6. *Measurement of tumor burden (growth) in mice using the IVIS Spectrum animal imaging system*

Mice were anesthetized using inhaled isoflurane, then injected intraperitoneally with 3 mg of luciferin in 100 µL PBS. After 10 minutes, to reach a pre-established luciferase signal plateau, three mice were placed on their backs on the stage of the Lumina II imaging system (Perkin-Elmer), where anesthesia was maintained by the onboard isofluorane system. Radiance of the image is expressed as photons/second. White light and bioluminescence images were captured using the Living Image software with auto exposure, binning factor 8, field of view 12.5 cm, subject height 1.5 cm, F-stop 1 (*16*). After imaging the mice were removed from the inhaled anesthetic and allowed to recover. Animals were imaged once per week for four weeks prior to euthanasia. For detail of imaging the

Control mouse
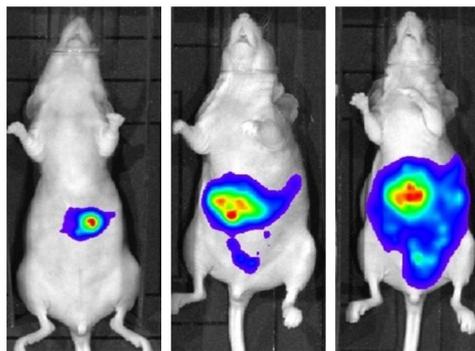
15K-treated mouse
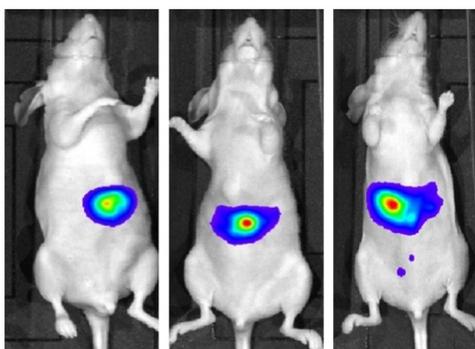
2 W   3 W   4 W

Figure 1. Imaging the luciferase-labeled cancer xenografts in mice (control *vs* 15K-treated). Top: an AsPC-1-bearing mouse (control); at 2, 3 and 4 weeks, from left to right. **Bottom:** an AsPC-1-bearing mouse treated with 15K (5 mg/kg); at 2, 3, and 4 weeks, from left to right. 15K clearly suppresses both growth and metastasis of the GEM-resistant pancreatic cancer during 3-4 weeks after the graft.

luciferase-labeled cancer xenografts in mice (control *vs* 15K-treated), see Figure 1.

2.7. *Autopsy and histological analysis*

Animals were anesthetized with ketamine/xylazine as described above for surgery. Blood was taken from the vena cava for hematological analysis and peritoneal organs and heart and lungs removed for histological analysis.

Tissues from autopsy were fixed in 10% formalin, dehydrated in increasing concentrations of ethanol, cleared with xylene and embedded in paraffin (Thermo Shandon Ltd, Cheshire, UK). 3-5 µm sections were prepared from the paraffin blocks using Shandon Finesse 325 microtome (Thermo Scientific Ltd., Cheshire, UK) and stained with hematoxylin and eosin (Thermo Shandon Ltd., Cheshire,UK), dehydrated in ascending concentrations of ethanol, cleared in xylene and mounted in DPX (Sigma Aldrich Steinheim,Germany). Histologic evaluation was performed under light microscopy.

2.8. *Statistical analysis*

The effects of 15K on growth of AsPC-1 and BxPC-3 tumors in athymic mice, measured as total flux (radians/sec) was analysed by two-way analysis of variance with drug dose and time as the variables and with Bonferroni post-hoc tests for the different time points. Statistical analysis of the occurrence of metastases was carried out using Fisher's exact test. Data analysis was performed using SPSS software (IBM).

3. Results

3.1. *Effects of 15K on the growth of cultured human pancreatic cancer cells*

Addition of 15K to the culture media caused a concentration-dependent decrease in viable cell number in all three GEM-resistant cancer cell lines (see Figure 2). The potency of 15K was greater in AsPC-1 and S2013 cells compared with that in BxPC-3 cells as judged by the IC_{50} for 15K in these cell lines (AsPC-1: 41 nM; BxPC-3: 88 nM; and S2013: 52 nM).

3.2. *Anti-pancreatic cancer activity of 15K in orthotopically transplanted pancreatic cancers in athymic mice*

Administration of 15K caused statistically significant, dose-responsive inhibition of total tumor burden (growth) in animals with either AsPC-1 (ANOVA: $p < 0.0001$, Dose $F = 8.78$, Df = 3; Time $F = 28.4$, Df = 3; Interaction $F = 4.28$, Df = 9) or BxPC-3 (ANOVA: $p < 0.0001$, Dose $F = 11.73$, Df = 3; Time $F = 19.30$, Df = 3; Interaction $F = 6.35$, Df = 9) orthotopic transplants (see Figure 3).

During 4 weeks, 15K had reduced total burden of AsPC-1 tumors by 53%, 69% and 85% with daily doses
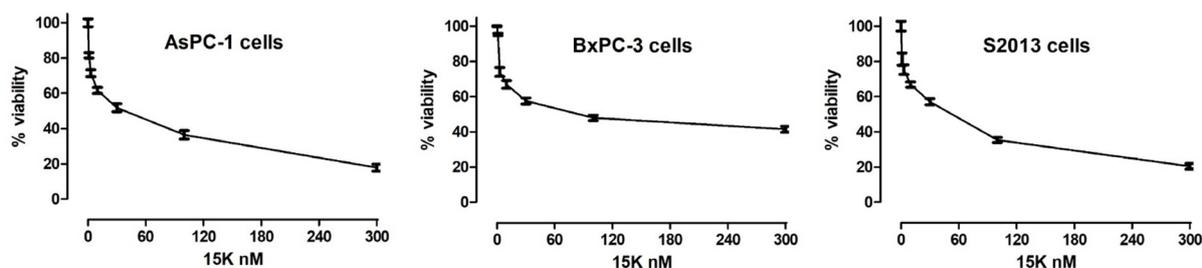

Figure 2. Effect of 15K on the growth/viability of human pancreatic cancer cell lines. Left: AsPC-1; **Middle:** BxPC-3; and **Right:** S2013. Each cell line was treated with 15K in serum free conditions at given concentrations for 72 h, and its viability was monitored by counting viable cell number. Results are expressed as percentage of control untreated cells.
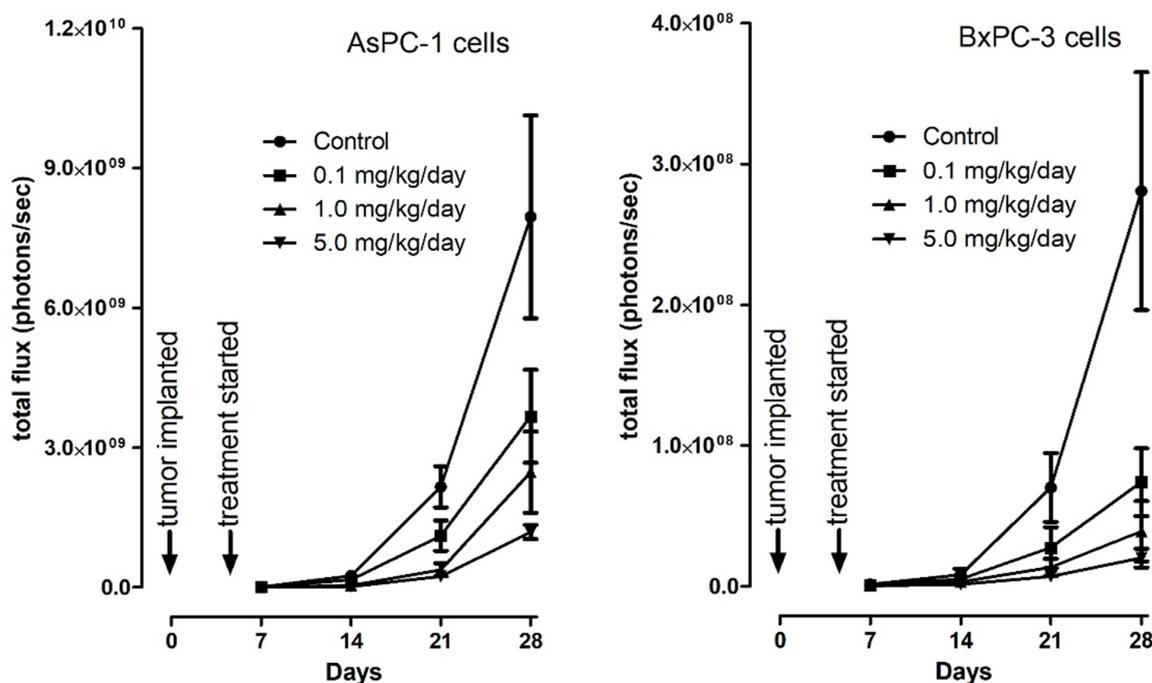

Figure 3. Effect of 15K on total tumor burden of GEM-resistant human pancreatic cancer xenograft in mice. Left: Female mice with AsPC-1 xenografts; **Right:** male mice with BxPC-3 xenografts. Total tumor burden was measured by bioluminescence (photons/sec) at weekly intervals after transplant.

Table 1. Number of female mice with AsPC-1 cancer xenografts showing tissue tumor invasion or metastases. Table shows numbers of animals and % in parentheses

Groups	Control, 0 mg/kg/day	15K, 0.1 mg/kg/day	15K, 1 mg/kg/day	15K, 5 mg/kg/day	Significance
Number of Mice	10	10	8	10	
Pancreas	10 (100%)	10 (100%)	6 (75%)	5 (50%)	NS
Abdominal Wall	8 (80%)	8 (80%)	4 (50%)	4 (40%)	NS
Esophagus	6 (60%)	3 (30%)	1 (12.5%)	0 (0%)	$P < 0.05$
Stomach	9 (90%)	3 (30%)	4 (50%)	3 (30%)	NS
Small Intestine	8 (80%)	8 (80%)	5 (62.5%)	4 (40%)	NS
Colon	0 (0%)	1 (10%)	1 (12.5%)	0 (0%)	NS
Liver	9 (90%)	6 (60%)	0 (0%)	1 (10%)	$P < 0.01$
Spleen	3 (30%)	2 (20%)	1 (12.5%)	0 (0%)	NS
Kidney	7 (70%)	1 (10%)	1 (12.5%)	1 (10%)	NS
Lung	2 (20%)	4 (40%)	1 (12.5%)	0 (0%)	NS
Lymph Nodes or LVI	9 (90%)	7 (70%)	7 (87.5%)	3 (30%)	NS
Peritoneum	10 (100%)	8 (80%)	6 (75%)	4 (40%)	NS

Table 2. Number of male mice with BxPC-3 cancer xenografts showing tissue tumor invasion or metastases. Table shows numbers of animals and % in parentheses

Groups	Control, 0 mg/kg/day	15K, 0.1 mg/kg/day	15K, 1 mg/kg/day	15K, 5 mg/kg/day	Significance
Number of Mice	8	10	9	8	
Pancreas	8 (100%)	9 (90%)	4 (44.4%)	2 (25%)	NS
Abdominal Wall	8 (100%)	4 (40%)	4 (44.4%)	2 (25%)	NS
Esophagus	4 (50%)	2 (20%)	2 (22.2%)	0 (0%)	NS
Stomach	8 (100%)	6 (60%)	4 (44.4%)	2 (25%)	NS
Small Intestine	3 (37.5%)	3 (30%)	1 (11.1%)	2 (25%)	NS
Liver	5 (62.5%)	1 (10%)	1 (11.1%)	0 (0%)	$P < 0.05$
Spleen	2 (25%)	0 (0%)	0 (0%)	0 (0%)	NS
Kidney	2 (25%)	3 (30%)	2 (22.2%)	0 (0%)	NS
Heart	0 (0%)	1 (10%)	0 (0%)	0 (0%)	NS
Lymph Nodes or LVI	5 (62.5%)	4 (40%)	1 (11.1%)	1 (12.5%)	NS
Peritoneal Invasion	4 (50%)	2 (20%)	3 (33.3%)	0 (0%)	NS

of 0.1, 1 and 5 mg/kg, *i.p.*, respectively (see Figure 1 and Figure 3, left, all $p < 0.001$). Similarly, with the same corresponding doses, 15K had reduced total burden of BxPC-3 tumors by 74%, 86% and 93%, respectively (see Figure 3, right, all $p < 0.001$). Thus, in both cases, the *in vivo* IC$_{50}$ of 15K against the growth of these human pancreatic cancers was below 0.1 mg/kg/day.

3.3. *Effect of 15K on both tumor invasion and metastasis*

The results of histological analysis showing the presence of tumor cells in the pancreas and other tissues of AspC-1 and BxPC-3 transplanted animals are shown in Tables 1 and 2, respectively. Tumor burden observed grossly and microscopic was larger in the AsPC-1 cancer xenografts mice in comparison to those seen in the BxPC-3 cancer xenografts mice. In the majority of the control animals, with both cell lines, the pancreatic tumor had invaded into the serosal surface of the stomach and/or the small intestine, as well as the abdominal wall adjacent to the laparotomy scar. The latter invasion was presumably triggered by the growth factor responses from the healing tissue. There is a possibility that seeding occurred during the surgical procedure in some animals, but no leakage was apparent from the injection site. Indeed, a macroscopically well-defined bubble was evident in the duodenal lobe of the pancreas of all transplanted animals. Imaging immediately after transplantation would have revealed any leakage, this was intended by unfortunately could not be carried out because the IVIS imaging system was not working at that time.

AsPC-1 Cancer Xenografts: All female mice with AsPC-1 cancer xenografts in the control group had tumor in the pancreas and peritoneum. The majority had tumor present in organs adjacent to the pancreas. The serosal surface +/– variable thickness of wall was involved in stomach (9/10), duodenum/small intestine (8/10) and esophagus (6/10). Tumor was present in 7/10 control mice in the peri-renal adipose tissue in the form of tumor nodules without invasion of the renal or adrenal parenchyma except for one case that had parenchymal invasion. Metastasis was present in the liver (9/10), pelvic lymph nodes (5/10), peri-tumoral lymph nodes (4/10), spleen (3/10), and lung (2/10) as well, but no tumor was detected in the uterus or ovaries in the control or treated mice. Lymph-vascular invasion and/or lymph node metastasis was present in 9/10 (90%) of AsPC-1 control cases. The frequency decreased from

70% to 30% with increasing dose of 15K. The tumor was detected in the pancreas, peritoneum, stomach and adjacent small intestine and esophagus with a decreased frequency as the dose of 15K increased in both the AsPC-1 (Table 1), however this reduction only reached statistical significance in the liver and esophagus. Two animals with AsPC-1 tumors had lung micro-metastases. In two animals of the AsPC-1, 5 mg/kg group, no tumor was detected in any organ including the pancreas and the adjacent peritoneal/adipose tissue or stomach/duodenum or the surgical scar. However, in one case, tumor mass was detected around the sternum. In the other mouse, no tumor was detected in any organ. The histological observations were confirmed by the imaging which showed lack of tumor burden in these cases. This could reflect the effect of high dose 15K in these animals. In three cases from this 15K-treated group where tumor was no longer seen in the pancreas, it was detected in adjacent tissue including stomach, duodenum or peritoneum.

BxPC-3 Cancer Xenografts: All male mice with BxPC-3 cancer xenografts in the control group had tumor in the pancreas stomach and abdominal wall. Tumor was present in small intestine (37.5%), esophagus (50%), liver (62.5%), spleen (25%), and surface of kidney (25%). Peritoneal tumor nodules were present in 50% of the control group. Tumor detected in the pancreas decreased in frequency from 90% in the 0.1 mg/kg to 44% and 25% in the 1 and 5 mg/kg groups, respectively. Similarly, there was a reduction in tumors detected in the abdominal wall and stomach from 40% and 60% in the 0.1 mg group to 25% and 25% in the 5 mg/kg group, respectively.

Tumor was detected in the spleen of 20% of untreated animals but was not detected in the spleen in any of the treated animals. Tumor was present in the liver in 50% of untreated animals and this decreased frequency of 10%, 10% and 0% as the dose of 15K increased.

Lymph-vascular invasion and/or lymph node metastasis was detected in 5/8 (62.5%) of BxPC-3 control cases. The frequency decreased from 40% to 12.5% with increasing dose of 15K. No tumor metastasis was found in either testis, colon or heart of the control group. Tumor detected in the pancreas, peritoneum, liver, stomach and adjacent small intestine, esophagus, kidney with a decreased frequency as the dose of 15K increased in both the BxPC-3 mice (Table 2), however this reduction only reached statistical significance in the liver.

Tumor presence in liver and kidney with both cell lines was mostly found on the surface of these tissues, suggestive of peritoneal seeding rather than vascular invasion. Indeed, nine of the AsPC-1 group and four of the BxPC-3 group had peritoneal metastases, supporting tumor seeding through this route. Numbers of animals with local invasion and metastatic spread were lower in the treated groups in a dose-dependent pattern and generally tumor volumes were clearly lower in treated animals as reflected in the total tumor burden. One animal in the high dose AsPC-1 group and four animals in the high dose BxPC-3 group were tumor-free at autopsy.

3.4. *No effect of 15K on body weight, and hematological parameters*

The tested doses of 15K on mice, up to 5 mg/kg/day, were well-tolerated as evidenced by the lack of any side effect on either body weight throughout the experiment or hematological parameters measured at the time of euthanasia. Body weights measured at weekly intervals (see Figure S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=47*). There were no significant changes in haemoglobin, red cell counts, white cell counts or platelet counts between the two groups (see Figure S2, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=47*). Hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular haemoglobin concentration were also no different between the groups (data not shown).

4. Discussion

First of all, orthotopical graft of luciferase-expressing cancer cell lines on pancreas was quite helpful for non-invasive (luminescence-based) weekly quantification of the cancer mass growth or burden. The main reason why we have chosen the orthotopical graft (instead of technically far easier subcutaneous graft) of these cancers is that the latter approach prevents pancreatic cancers from significant metastases. Injection of cancer cells into tail vein was not our choice for metastasis model, simply because it is very far from human conditions. Nevertheless, we managed to confirm the extremely potent anti-mitotic and anti-metastatic property of 15K *in vivo* (against xenograft of human GEM-resistant pancreatic cancers in mice).

In the past, several distinct anti-cancer drugs including a few PAK1 inhibitors have been shown to overcome the GEM-resistance of human pancreatic cancers *in vivo*. For example, frondoside A (FRA) from a sea cucumber, that directly inhibits PAK1 with IC_{50} around 1 μM (*17*), inhibits the growth of GEM-resistant human pancreatic cancer cells (AsPC-1, S2013, MiaPaCa2) in culture with the same IC_{50}. Furthermore, frondoside A inhibits the growth of pancreatic cancer xenografts of AsPC-1 and S2013 cells in mice with an IC_{50} below 100 μg/kg/day (*18*). Another natural PAK1-blocker called triptolide or its prodrug (phosphorylated derivative) called minnelide inhibited the growth of GEM-resistant human pancreatic cancer xenografts in mice with the IC_{50} around 0.3 mg/kg/day (*19*).

Among the synthetic PAK1-blockers, YM155 has been the most potent, suppressing survivin expression by blocking PAK1 signalling, but inhibits the growth of GEM-resistant human pancreatic cancer *in vivo* with IC$_{50}$ around 10 mg/kg/day (*20*). Thus, to the best of our knowledge, so far 15K appears to be among the most potent PAK1-blockers that suppress both growth and metastasis of GEM-resistant human pancreatic cancers *in vivo* without apparent side effects. Interestingly, among these potent PAK1-blockers, 15K is able to extend the healthy lifespan of *C. elegans* at 50 nM (*7*), while triptolide needs over 1000 times higher concentrations (around 140 μM) to show a similar lifespan extending effect (*21*). Moreover, 15K has a great advantage in the mass production for clinical use over natural PAK1-blockers, because its starting material (ketorolac) is a generic synthetic pain-killer developed and sold more than three decades ago (*4,5*). Thus, we look forward to commencing its clinical trials for GEM-resistant pancreatic cancers shortly.

Finally, it is worth noting that the "brand-new" role of BETs (bromodomain and exterminal domain proteins) was recently identified. BETs are responsible for expression of both the receptor PD-1 in T-cells and its ligand PD-L1 in cancer cells *via* β-catenin-MYC pathway (*22*), which eventually destroys our "immune-surveillance" of cancers. Furthermore, a few years ago, a series of 1,2,3-triazol compounds have been found to inhibit BETs (*23*). Since 15K is also among 1,2,3-triazol compounds, it is most likely that 15K suppresses both PD-1 and PD-L1 by blocking not only PAK1 but also BETs, both of which are essential for the activation of the oncogenic β-catenin-MYC pathway. Thus, 15K could be a more effective (safer and less expensive as well) therapeutic than the current monoclonal-based immune (check-point) therapeutics which have never worked for the treatment of brain tumors and pancreatic cancers (*24*).

References

1. Maruta H, Ahn MR. From bench (laboratory) to bed (hospital/home): How to explore natural and synthetic PAK1-blockers/Longevity-promoters for cancer therapy, Eur J Med Chem. 2017; 142:229-243.
2. Jagadeeshan S, Venkatraman G, Rayala SK. Targeting p21 activated kinase 1 (Pak1) to PAKup pancreatic cancer. Expert Opin Ther Targets. 2016; 20:1283-1285.
3. Hirokawa Y, Levitzki A, Lessene G, Baell J, Xiao Y, Zhu H, Maruta H. Signal therapy of human pancreatic cancer and NF1-deficient breast cancer xenograft in mice by a combination of PP1 and GL-2003, anti-PAK1 drugs (Tyr-

kinase inhibitors). Cancer Lett. 2007; 245:242-251.
4. Nguyen BCQ, Takahashi H, Uto Y, Shahinozzaman MD, Tawata S, Maruta H. 1,2,3-Triazolyl ester of Ketorolac: A "click chemistry"-based highly potent PAK1-blocking cancer-killer. Eur J Med Chem. 2017; 126:270-276.
5. Vit JP, Ohara PT, Tien DA, Fike JR, Eikmeier L, Beitz A, Wilcox GL, Jasmin L. The analgesic effect of low dose focal irradiation in a mouse model of bone cancer is associated with spinal changes in neuro-mediators of nociception. Pain. 2006; 120:188-201.
6. Guo Y, Kenney Jr SR, Muller CY, Adams S, Rutledge T, Romero E, Murray-Krezan C, Prekeris R, Sklar LA, Hudson LG, Wandinger-Ness A. R-ketorolac targets Cdc42 and Rac1 and alters ovarian cancer cell behaviors critical for invasion and metastasis. Mol Cancer Ther. 2015; 14:2215-2227.
7. Nguyen BC, Kim SA, Won SM, Park SK, Uto Y, Maruta H. 1,2,3-Triazolyl ester of ketorolac (15K): Boosting both heat-endurance and lifespan of *C. elegans* by down-regulating PAK1 at nM levels. Drug Discov Ther. 2018; 12:92-96.
8. Deer EL, Gonzalez-Hernandez J, Coursen JD, Shea JE, Ngatia J, Scaife CL, Firpo MA, Mulvihill SJ. Phenotype and genotype of pancreatic cancer cell lines. Pancreas. 2010; 39:425-435.
9. Tan MH, Nowak NJ, Loor R, Ochi H, Sandberg AA, Lopez C, Pickren JW, Berjian R, Douglass HO Jr, Chu TM. Characterization of a new primary human pancreatic tumor line. Cancer Invest. 1986; 4:15-23.
10. Fredebohm J, Boettcher M, Eisen C, Gaida MM, Heller A, Keleg S, Tost J, Greulich-Bode KM, Hotz-Wagenblatt A, Lathrop M, Giese NA, Hoheisel JD. Establishment and characterization of a highly tumourigenic and cancer stem cell enriched pancreatic cancer cell line as a well-defined model system. PLOS One. 2012; 7:e48503.
11. Amrutkar M, Gladhaug IP. Pancreatic cancer chemoresistance to gemcitabine. Cancers. 2017; 9:E57.
12. Arlt A, Gehrz A, Muerkoster S, Vorndamm J, Kruse ML, Folsch UR, Schafer H. Role of NF-kappaB and Akt/PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death. Oncogene. 2003; 22:3243-3251.
13. Rathos MJ, Joshi K, Khanwalkar H, Manohar SM, Joshi KS. Molecular evidence for increased antitumor activity of gemcitabine in combination with a cyclin-dependent kinase inhibitor, P276-00 in pancreatic cancers. J Transl Med. 2012; 10:161.
14. Al Shemaili J, Mensah-Brown E, Parekh K, Thomas SA, Attoub S, Hellman B, Nyberg F, Adem A, Collin P, Adrian TE. Frondoside A enhances the antiproliferative effects of gemcitabine in pancreatic cancer. Eur J Cancer. 2014; 50:1391-1398.
15. Hennig R, Ventura J, Segersvard R, Ward E, Ding XZ, Rao SM, Jovanovic BD, Iwamura T, Talamonti MS, Bell RH Jr, Adrian TE. LY293111 improves efficacy of gemcitabine therapy on pancreatic cancer in a fluorescent orthotopic model in athymic mice. Neoplasia. 2005; 7:417-425.
16. Lim E, Modi KD, Kim J. *In vivo* bioluminescent imaging of mammary tumors using IVIS Spectrum. J Vis Exp. 2009; 26:e1210.
17. Nguyen BCQ, Yoshimura K, Kumazawa S, Tawata S, Maruta H. Frondoside A from sea cucumber and nymphaeols from Okinawa propolis: Natural anti-cancer agents that selectively inhibit PAK1 *in vitro*. Drug Discov

Ther. 2017; 11:110-114.

18. Adrian TE, Collin P. The anti-cancer effects of frondoside A. Mar Drugs. 2018; 16:E64.

19. Chugh R, Sangwan V, Patil SP, Dudeja V, Dawra RK, Banerjee S, Schumacher RJ, Blazar BR, Georg GI, Vickers SM, Saluja AK. A preclinical evaluation of minnelide as a therapeutic agent against pancreatic cancer. Sci Transl Med. 2012; 4:156ra139.

20. Zhao X, Puszyk WM, Lu Z, Ostrov DA, George TJ, Robertson KD, Liu C. Small molecule inhibitor YM155-mediated activation of death receptor 5 is crucial for chemotherapy-induced apoptosis in pancreatic carcinoma. Mol Cancer Ther. 2015; 14:80-89.

21. Kim SJ, Beak SM, Park SK. Supplementation with triptolide increases resistance to environmental stressors and lifespan in *C. elegans*. J Food Sci. 2017; 82:1484-1490.

22. Andrieu GP, Shafran JS, Smith CL, Belkina AC, Casey AN, Jafari N, Denis GV. BET protein targeting suppresses the PD-1/PD-L1 pathway in triple-negative breast cancer and elicits anti-tumor immune response. Cancer Lett. 2019. pii: S0304-3835(19)30450-1.

23. Sharp PP, Garnier JM, Hatfaludi T, *et al*. Design, synthesis, and biological activity of 1,2,3-triazolobenzodiazepine BET bromodomain inhibitors. ACS Med Chem Lett. 2017; 8:1298-1303.

24. Maruta H. Breakthrough: PAK1-dependent expression of PD-L1 (programmed death ligand). Integr Mol Med. 2019; 6:1-2.