RP101 improves the efficacy of chemotherapy in pancreas carcinoma cell lines and pancreatic cancer patients

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RP101 [(*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU)], which supports apoptosis and prevents the acquisition of chemoresistance, was tested in cultured human pancreatic tumor cells. RP101 downregulated uridine phosphorylase, a marker of poor prognosis, and APEX1, which is involved in DNA repair, and repressed Stat3 and its target vascular endothelial growth factor. Furthermore, RP101 activated antitumor immunity as demonstrated by enhanced cytolytic activity of NK-92 natural killer cells. This was concomitant with an enhanced expression of lymphotoxins α and β, natural killer cell transcript 4, tumor necrosis factor LIGHT/TNFSF-14, and intercellular adhesion molecule-1 in pancreas carcinoma cells. These results encouraged us to investigate the effect of RP101 in pancreas cancer patients. Here, we present data from two RP101 combination therapy schemes. In a first pilot study, 13 patients in stage III and VI of the disease were treated with gemcitabine + cisplatin + RP101. RP101 co-treatment enhanced remissions, survival and time to progression. Seventy-seven percent of the patients lived or have lived longer than 1 year, and 23% have lived more than 2 years. Median survival was 447 days, time to progression 280 days and the response rate 33%. A second study with 21 patients in similar stages of disease, treated with RP101 + gemcitabine alone, confirmed the results of the pilot study. Eighty-three percent of the presently evaluable patients live or lived 0.5 years or longer and 33% 1 year or longer. Considering both studies, the tumor control was 94%. The data indicate that acquisition of chemoresistance was prevented and the antitumor efficacy of standard chemotherapy was improved. To our knowledge, RP101 co-treatment is more efficient than any other regimen published. *Anti-Cancer Drugs* 17:1045–1056 © 2006 Lippincott Williams & Wilkins.

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Introduction

In preclinical studies, RP101 [(*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU)] has shown strong antitumor effects due to inhibition of chemoresistance and the enforcement of apoptotic response upon cancer drug treatment [1]. RP101 affects numerous gene products related to chemoresistance and tumor immunity, such as:

(1) inhibition of oncogenic and DNA repair-associated components,
(2) suppression of chemotherapy-induced *MDR1* (multi-drug resistance) or *DHFR* (dihydrofolate reductase) gene amplification,
(3) repression of survival pathways,
(4) induction of NQO1 [NAD(P)H–quinone oxido-reductase 1] activity, and
(5) repression of ATP-generating enzymes during recovery phase [1].

The present study had two aims. (1) Further in-vitro elucidation of the mode of action of RP101 using cultured pancreas tumor cells. For ethical reasons, tumor probes of patients were not available. (2) Performance of two independent clinical studies with metastasized pancreas cancer patients to evaluate the efficacy of the treatment.

In a preceding phase I study including 31 patients with five different tumor entities and 12 different cytostatic drugs, no enhancement of unwanted side-effects had been observed [2]. During this study, two partial remissions occurred in four stage IV pancreas cancer patients, who were treated with gemcitabine (GEM) + cisplatin (CIS) + RP101. For this reason, we increased the group of pancreas cancer patients to 13 cases, which are the focus of the pilot study presented here. As control, we used data from patients of a preceding study who were treated with GEM and CIS, but not with

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RP101 (Heinemann study) [3]. With the intention to obtain a control group with a similar proportion of stage IV and stage III diseases compared with the 13 patients of the pilot study, 22 of 96 patients were selected from the Heinemann study by a random generator.

As GEM + CIS is not the standard treatment for pancreas cancer patients, the second study was performed with GEM in combination with RP101, but without CIS. In this study, five different doses of RP101 were used. The first four patients were treated with the same dose as in the pilot study. The RP101 dose was increased stepwise so that the last four patients received a double dose. Of the 22 treated patients, one was excluded. A patient group (Heinemann study) of identical size, and with a similar proportion of stage IV and stage III diseases served as a control group, which was treated with GEM alone.

Methods

Chemicals

GEM was purchased from Eli Lilly Deutschland (Bad Homburg, Germany) and RP101 was from RESprotect (Dresden, Germany). All of the other chemicals were from Sigma-Aldrich (Taufkirchen, Germany) and Roth (Karlsruhe, Germany), unless otherwise indicated.

Human pancreas adenocarcinoma cell lines

AsPC-1 and BxPC-3 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin, streptomycin and 1 mmol/l sodium pyruvate (Biochrom, Berlin, Germany). CAPAN-2 cells were grown in RPMI 1640 medium supplemented with 15% fetal bovine serum, penicillin and streptomycin. Cells were seeded out at a density of 24 000 cells/cm^2 and treated with increasing doses of GEM (typically 2, 5, 8 and 12 ng/ml) with and without RP101 (30 μmol/l). At about 80% confluence, cells were trypsinized and replated at the next higher drug concentration. The number of living cells was determined using the Cell Counter and Analyzer System CASY TT (Schärfe System Reutlingen, Germany).

NK-92, human natural killer lymphoma

Cells were grown in suspension in 75% α-minimum essential medium + 12.5% fetal bovine serum + 12.5% horse serum + 2 mmol/l L-glutamine + 10 ng/ml interleukin-2 (ProSpec-Tany TechnoGene, Rehovot, Israel).

Natural killer cell assay: morphometric analysis of cytolysis in cultured cell monolayers

CAPAN-2 cells were treated with increasing doses of GEM (2, 5, 8 and 12 ng/ml) with and without RP101 (30 μmol/l) as described above. Cells were grown to confluence, GEM was removed and NK-92 natural killer cells were inoculated on top of the monolayer in a ratio of 4 : 1 (NK-92 : CAPAN-2) in the appropriate medium (75% α-minimum essential medium + 12.5% fetal bovine serum + 12.5% horse serum + 2 mmol/l L-glutamine + 10 ng/ml interleukin-2) with or without RP101. After 12–48 h, when lysis of the target cells became visible as clear areas in the target monolayer, nonadherent cells were washed off, and intact adherent cells were fixed and stained with a Coomassie blue solution [0.2% Brilliant blue G, (Sigma B0770) in acetic acid/methanol/water (10/45/45: v/v/v)]. Images were taken using an 'HP Scanjet 4400c' (Hewlett-Packard, Böblingen, Germany) or a Canon Power Shot G5 digital camera (Canon Deutschland, Krefeld, Germany) connected to a Zeiss Axiovert 25 microscope (Carl Zeiss Jena, Jena, Germany). Intact adherent cells stained blue when exposed to Coomassie blue solution while unstained areas became visible after target cell lysis. To quantify lysis, clearance in the target monolayer was determined using the histogram function of the NIH ImageJ software [4].

Western blot analysis

Protein extraction was performed with pelleted cells according to Pagano as described recently [1]. Protein concentration was estimated on a Tecan Multireader (Gröding, Salzburg, Austria) using the Bradford method. Whole-cell lysates (5–10 μg) were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred on a polyvinylidene difluoride membrane (Amersham Biosciences, Freiburg, Germany). Membranes were blocked with 10% Roti-Block (Rothmany) in TBST and incubated overnight with antibodies against Stat3 (1 : 2500), phospho-Stat3-Y705 (1 : 2500; both from NEB, Frankfurt a.M., Germany) or against APEX1 (1 : 10 000; Santa Cruz Biotechnology, Santa Cruz, California, USA). As secondary antibodies, horseradish peroxidase conjugates (NEB) were used and their binding was visualized using Chemoluminescence Reagent Plus (Perkin Elmer, Boston, Massachusetts, USA) on a Kodak Image Station 440CF. Analysis was performed with 1d Image Analysis Software (v3.5) (Eastman-Kodak, Rochester, New York, USA).

Molecular and cell biology

Unless otherwise indicated, all molecular procedures were performed as described recently by Fahrig *et al.* [1] or by Sambrook and Russell [5].

Quantitative real-time polymerase chain reaction

Total RNA was isolated from each cell subline at various time points using the RNeasy Mini Kit (Qiagen, Hilden, Germany), cDNA was synthesized using the ImProm-II Reverse Transcription System (Promega, Madison, Wisconsin, USA). Quantitative real-time polymerase chain reaction (PCR) was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, California, USA). Primers and TaqMan

fluorogenic probes were used as follows: uridine phosphorylase (UPase) forward primer, 5′-GCT GCT GTA CTA TGC CCG GT-3′, UP reverse primer, 5′-TCT GTT ATG ACC ACA GTG CCG-3′, UP TaqMan probe, 5′-TCC GCA TTG GCA CTT CTG GTG G-3′; natural killer cell transcript 4 (NK4) forward primer, 5′-ACG TGG ACA GGT GAT GTC GA-3′, NK4 reverse primer, 5′-AAG CCG CCA CTG TCT CCA G-3′, NK4 TaqMan probe, 5′-TGG CAG AGC TGG AGG ACG ACT TCA-3′; lymphotoxin-β (LTB) receptor (LTBR) forward primer, 5′-CAC AAG CAA ACG GAA GAC CC-3′, LTBR reverse primer, 5′-TTG AGC TCG GCT TCA GTG C-3′, LTB receptor TaqMan probe, 5′-TGC CAG CCG GGA ATG TTC TGT G-3′; LTB forward primer, 5′-TGC TAG CTG TGG CAG GAG C-3′, LTB reverse primer, 5′-CAG CAC AGC CAG GAC AGT GA-3′, LTB TaqMan probe, 5′-TGA CCT TGT TGC TGG CGG TGC C-3′; LIGHT forward primer, 5′-TGG TAC CCG GTC TTA CTT CGG-3′, LIGHT reverse primer, 5′-GGC ACC CTC TGA GTT CTC CA-3′, LIGHT TaqMan probe, 5′-AGC GTG GTG CAT TGG ACA TGG GT-3′; lymphotoxin-α (LTA) forward primer, 5′-TCC ATT CTG ACC ATT TCA GGG-3′, LTA reverse primer, 5′-CGG TGA CTT GAT CAG GGA AGA-3′, LTA TaqMan probe, 5′-TCG TCA CCA CCT CTC CTT TGG CCA-3′; APEX1 forward primer, 5′-TGG TTG GCG CCT TGA TTA CT-3′, APEX1 reverse primer, 5′-TGG AAC GGA TCT TGC TGT CA-3′, APEX1 TaqMan probe, 5′-TGT TGT CCC ACT CTC TGT TAC CTG CAT TGT-3′; vascular endothelial growth factor (VEGF) forward primer, 5′-GCG CAA GAA ATC CCG GTA TAA-3′, VEGF reverse primer, 5′-ATG CTT TCT CCG CTC TGA GC-3′, VEGF TaqMan probe, 5′-TGG AGC GTT CCC TGT GGG CC-3′.

Patients

The ethical committees of the Technical University Dresden, the University of Munich, the Technical University Munich and the 'Bundesinstitut für Arzneimittel und Medizinprodukte' (BfArM), Berlin, have approved the clinical studies. We confirm that informed consent was obtained from all patients.

Inclusion criteria

Patients were enrolled into this protocol only if all of the following inclusion criteria were met: male or female patients of age between 18 and 75 years; patients with a histologically/cytologically proven pancreatic cancer and being eligible for anti-cancer therapy; Eastern Cooperative Oncology Group (ECOG) performance status 0, 1 or 2; life expectancy of at least 3 months; objectively measurable tumor parameters; if the patient was female and of child-bearing potential she had to have a negative β-human chorionic gonadotrophin level before receiving treatment; the patient had to practice a medically approved method of contraception during the study (all patients potentially fertile).

Exclusion criteria

Patients were excluded from enrolment if any of the following applied: any preceding cytotoxic chemotherapy; any of the following abnormal baseline hematological values: hemoglobin < 6.2 mmol/l (10 g/dl), white blood cell 3.0 Gpt/l (3.0×10^9/l), neutrophils < 2.0 Gpt/l (2.0×10^9/l), platelets < 50 Gpt/l (50×10^9/l); any of the following abnormal baseline liver function tests: serum bilirubin > 42.8 µmol/l (2.5 mg/dl), ALAT and/or ASAT $> 2.5\times$ upper normal limit (unless due to liver metastasis where ALAT and ASAT could be $> 2.5\times$ upper normal limit), serum creatinine > 132.6 µmol/l (1.5 mg/dl). Withdrawal of consent without progression before two cycles were finished was a further exclusion criterion.

Treatment protocol

Pilot study

Thirteen patients (mean age 61, 69% males, four stage III, nine stage IV) with advanced pancreatic adenocarcinoma were treated with i.v. 1.000 mg/m^2 GEM over 30 min on days 1 and 15 of a 28-day schedule. CIS was administered at 50 mg/m^2. RP101 treatment took place on the same day and for 4 days after chemotherapy (each four 125-mg tablets per day, resulting in a total dose of 6.000 mg per treatment cycle). As control we used data from patients of a preceding study which were treated with GEM and CIS, but not with RP101 [3]. With the intention to obtain a control group with a similar proportion of stage IV and stage III diseases as the 13 patients of the pilot study, *22* (six stage III, 15 stage IV) of 96 patients were selected from the Heinemann study by a random generator.

Dose finding study

Twenty-two patients with advanced pancreatic adenocarcinoma were eligible for treatment in this single-arm study. The mean age was 60 years and 73% of patients were male. One patient without progressive disease was excluded from the study for withdrawal of consent after the first cycle. Of the 21 patients left, 15 were in stage IV and six in stage III. RP101 was administered in combination with GEM. On day 1, 8 and 15 of each 28-day cycle, RP101 was given together with GEM. On days 2–4, on days 8–11 and 15–18 of a 28-day cycle, RP101 was given alone. The starting dose of RP101 was 4×125 mg/day (total dose of 6.000 mg per treatment cycle) together with a fixed dose of GEM (1.000 mg/m^2). Subsequently, total doses of RP101 per cycle were 7500, 9000, 10500 and 12 000 mg, again in four patients per cohort.

First dose level: The starting dose of RP101 was 4×125 mg daily given at approximately 08:00 h (1×125 mg RP101), 12:00 h (1×125 mg RP101), 16:00 h (1×125 mg RP101) and 20:00 h (1×125 mg RP101). These doses were administered on days 1–4, 8–11 and 15–18. *Second dose level*: 1×250 mg + 3×125 mg daily. *Third dose level*: 2×250 mg + 2×125 mg daily. *Fourth dose level*: 3×250 mg + 1×125 mg daily. *Fifth dose level*: 4×250 mg daily.

Approximately 1 h after the 08:00-h RP101 administration on day 1, 8 and 15 of a cycle, the GEM infusion started and GEM was infused for exactly 30 min.

Patients were treated as long as there was no indication of tumor progression. A group of 21 patients (Heinemann study) treated with GEM alone, 15 being in stage IV and six in stage III of disease, served as a control group.

Analysis of the concentrations of gemcitabine and RP101 (BVDU) as well as analysis of the concentrations of BVU [(E)-5-(2-bromovinyl) uracil, degradation product of RP101] in plasma samples

Plasma (0.5 ml) spiked with about 0.5 μg of 4-propyl-2-thiouracil as internal standard was extracted with 2.5 ml of methanol-acetonitrile (v/v, 1 : 9) by vigorous mixing for about 1 min. After centrifugation, the supernatant was transferred to a beaker and evaporated to dryness at 50°C under a gentle stream of nitrogen. The residue was dissolved in 1 ml of the solution used as mobile phase by 10 min of treatment in an ultrasonic bath. After centrifugation, the clear supernatant was used for high performance liquid chromatography analysis. The concentrations of GEM and RP101 (BVDU) were calculated using a calibration curve with the respective standard substances. The concentrations of BVU were calculated using the calibration curve of RP101 and the determined response factor. Limits of detection were: RP101 (BVDU) 20 ng/ml plasma; GEM 30 ng/ml plasma. Limits of quantification were: RP101 (BVDU) 60 ng/ml plasma; GEM 100 ng/ml plasma.

Evaluation

Efficacy was measured by overall survival, objective tumor response rate (ORR, according to World Health Organization criteria in the pilot study and Response Evaluation Criteria in Solid Tumors (RECIST) criteria in the dose finding study) and time to progression (TTP) from the first administration onwards. Serum CA19-9 levels were measured at the start and end of each treatment cycle. Safety was evaluated according to NCI-CTC scale.

Evaluation of progression

Patients were evaluated every 5 weeks.

Statistical methods

Survival curves were generated using the Kaplan–Meier method; survival distributions were compared by the log-rank test (Cox–Mantel), and median survival by the Box and Whisker plot.

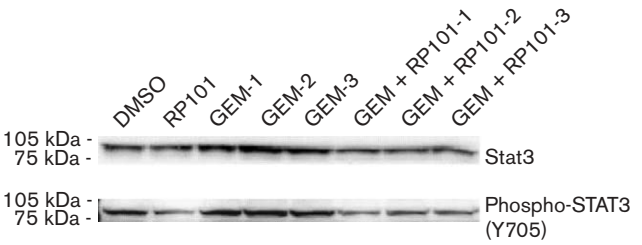
Results

Molecular effects of RP101 in human pancreas carcinoma cells

(1) The treatment of BxPC-3 pancreas carcinoma cells with the combination of RP101 + GEM prevented the upregulation of Stat3, which was induced by the singular treatment of GEM. Furthermore, phosphorylation of Stat3 at tyrosine 705 (Y705), which establishes the activity of Stat3, was repressed by RP101 (± GEM) even below constitutive levels (Fig. 1). The Stat3 target VEGF was downregulated by an average of 31% in pancreas carcinoma cells (Table 1).

(2) UPase gene expression seems strictly controlled by oncogenes, tumor suppressor genes and cytokines, and its activity is usually elevated in various tumor tissues. In pancreas carcinoma, UPase is a marker of poor prognosis. BxPC-3 and AsPC-1 human pancreas carcinoma cell lines were treated with dimethylsulfoxide (DMSO) (solvent control), RP101, GEM and GEM + RP101 for 30–50 days, and the isolated mRNA was subjected to quantitative reverse transcriptase (RT)-PCR analysis. Whereas RP101 alone did not exhibit a clear and predictive effect (compared with control), RP101 + GEM

Fig. 1



Effects of RP101 [(*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU)] on Stat3 protein expression and phosphorylation.

Table 1 Gene regulation as a result of RP101 treatment: quantitative real-time polymerase chain reaction

Gene regulation as a result of RP101 treatment	Human pancreas adenocarcinoma cell line	RP101 vs. DMSO control (%)	GEM + RP101 vs. GEM alone (%)
Uridine phosphorylase, Upase	BxPC-3	↔	↓ 66
	AsPC-1	↓ 64	↓ 73
Natural killer cell transcript 4, NK 4, IL-32	CAPAN-2	↑ 160	↑ 125
	AsPC-1	↑ 50	↑ 63
Lymphotoxin, β, LTB, tumor necrosis factor C	CAPAN-2	↑ 180	↑ 224
	AsPC-1	↑ 370	↑ 760
Lymphotoxin α, LTA, tumor necrosis factor:- β	CAPAN-2	↑ 20	↑ 76
	AsPC-1	↑ 70	↑ 94
Tumor necrosis factor LIGHT, TNFSF14	CAPAN-2	↑ 10	↑ 85
	AsPC-1	↑ 30	↑ 200
ICAM-1	CAPAN-2	↑ 32	↑ 54
	AsPC-1	↑ 53	↑ 135
VEGF, vascular endothelial growth factor	CAPAN-2	↓ 37	↓ 34
	AsPC-1	↓ 6	↓ 27
APEX, ref-1, apurinic endonuclease	CAPAN-2	↔	↓ 36

DMSO, dimethylsulfoxide; GEM, gemcitabine

substantially downregulated UPase expression by an average of 70% (see Table 1).

(3) Two different human pancreas carcinoma cell lines (CAPAN-2, AsPC-1) were treated with DMSO (solvent control), RP101, GEM and GEM + RP101 for 30–50 days, and the isolated mRNA was subjected to quantitative RT-PCR analysis. LTB was substantially induced by RP101 + GEM (2.2- to 7.6-fold) vs. GEM alone, but also by RP101 alone. To a lesser extent, LTA was induced by this treatment regime. The LTBR mRNA was weakly induced in the CAPAN-2 and AsPC-1 cell lines, whereas the receptor was strongly induced in a comparison experiment with rat AH13r Yoshida sarcoma cells (data not shown). NK4, tumor necrosis factor (TNF) LIGHT/TNFSF14, and intercellular adhesion molecule-1 (ICAM-1) were also enhanced upon exposure to RP101 (Table 1). The products of these modulated genes increase antitumor immunity.

(4) The endonuclease activity-bearing enzyme, APEX1 is part of the base excision repair system that restores DNA integrity in the case of damaged bases or abasic sites. Studies with antisense oligonucleotides revealed a significant enhancement of GEM toxicity in pancreatic cells, when APEX1 was suppressed [6]. In our experiments with CAPAN-2 cells, RP101 co-treatment inhibited overexpression of APEX1 (Table 1).

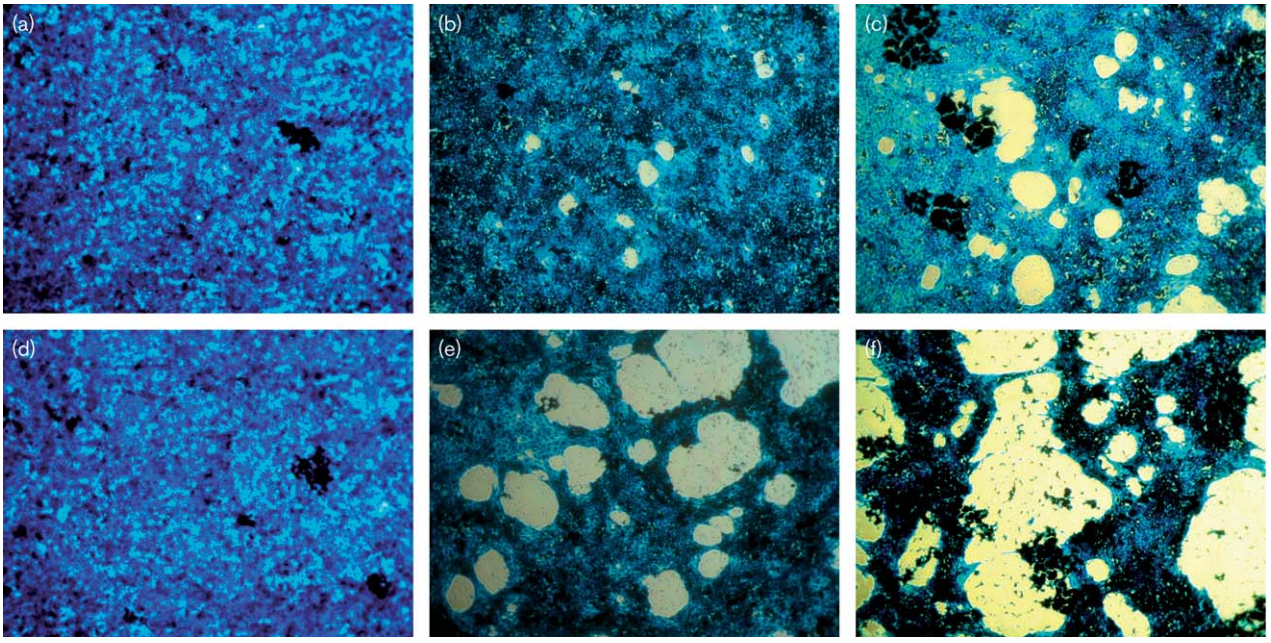
(5) RP101 treatment enhanced the cytolytic activity of NK-92 natural killer cells on CAPAN-2 cells (Fig. 2). Similar results were obtained with BxPC-3, YAPC and HT1080 cells (data not shown).

In summary, RP101 inhibited gene products that play a role in apoptosis (Stat3), define poor prognosis (UPase) or participate in DNA repair (APEX1). Furthermore, RP101 activated genes involved in antitumor immunity (LTA, LTB, NK4 and tumor necrosis factor LIGHT/TNFSF14) in human pancreatic tumor cell lines.

Clinical effects of RP101 in patients treated with GEM + CIS + RP101

All patients showed at least a stable disease and 33% of them showed remissions according to World Health Organization criteria (Table 2). The course of disease was followed by monitoring tumor marker CA19-9. The highest normal value is 35. In 75% of the cases, the tumor marker responded to the co-treatment (total dose of

Fig. 2



Effect of RP101 treatment on the cytolytic activity of NK-92 natural killer cells (NK) inoculated on top of CAPAN-2 cell monolayers. CAPAN-2 cell monolayers treated with RP101 for 14 days showed 2.3 times more cytolysis after 48 h of exposure to NK-92 cells in the presence of RP101 than untreated control cells (DMSO 0.05%). CAPAN-2 cell monolayers treated with GEM + RP101 for 18 days showed 2.9 times more cytolysis after 60 h of exposure to NK-92 cells in the presence of RP101 than cells treated with GEM alone. GEM was removed before NK-92 natural killer cells were added in a ratio of 4 : 1 (NK-92/CAPAN-2). Intact adherent cells stained blue when exposed to Coomassie blue solution while unstained areas became visible where target cells had been lysed by natural killer cells. (a) CAPAN-2 cells + GEM. (b) CAPAN-2 cells + NK-92 cells. (c) CAPAN-2 cells + GEM + NK-92 cells. (d) CAPAN-2 cells + GEM + RP101. (e) CAPAN-2 cells + RP101 + NK-92 cells. (f) CAPAN-2 cells + GEM + RP101 + NK-92 cells. RP101, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU); GEM, gemcitabine.

Table 2 Characteristics of patients treated with GEM + CIS + RP101

Patient no.	Age (years)	Stage	Survival (days)	Remissions sonography and CT	TTP (days)	Cycle RP101	CA19-9 before the first and after the sixth or earlier cycle
1	52	TxNxM1G2	447	partial	279	6	10621/155
2	59	TxNxM1G2	271	lung metastases	238	5	9372/7678
3	68	TxNxM0G4	549	SD	327	1	53/25
4	59	T4NxM1G2	>886	complete: liver metastases	735	4	1709/22
5	53	T4NxM1G2	80	SD	70	3	84/55101
6	63	TxNxMxG2	>880	partial	281	6	23362/21
7	63	T2N0M1Gx	407	minor	247	6	9436/117
8	64	TxNxM1G1	420	SD	331	1	1101/110
9	66	T3N1M0G2	447	partial	184	4	125/131
10	70	TxNxM1Gx	378	SD	285	6	739219/285
11	75	T4NxM1Gx	196	SD	102	3	7147/3734
12	41	T4NxMxG2	>789	partial (complete after surgery)	>789	4	332/6
13	63	TxNxM1Gx	463	SD	ND	2	ND
∅	61	4xIII, 9xIV	>478	33%	>322	4 (1–6)	75% PR or CR

GEM, gemcitabine; CIS, cisplatin; CT, computed tomography; TTP, time to progression; SD, stable disease; ND, no data; PR, partial remission; CR, complete remission.

RP101 per cycle was 6000 mg) and in several cases to the second-line therapy without RP101, indicating that the tumors had not acquired resistance (Fig. 3).

In the RP101 treatment group, median survival (447 days, $P = 0.006$) and TTP (280 days, $P = 0.004$) were significantly higher than that in the control group (Fig. 4). Ten of 13 patients (nine in stage IV and four in stage III) lived or have lived longer than 1 year after first treatment, and three of them have lived for more than 2 years by now. The patient characteristics are summarized in Table 2. In the control group [3], median survival was 186 days and median TTP 104 days (Fig. 4).

Clinical effects of RP101 in patients treated with GEM + RP101

Nineteen of 21 patients showed a stable disease, but only one a remission according to RECIST criteria (Table 3). The total doses of RP101 per cycle were 6000, 7500, 9000, 10 500 and 12 000 mg in four patients per group. The results are based on interim data from an ongoing study and patients without progression are still being treated. The data on the survival status show that 15 of 18 (83%) patients lived or have lived for 0.5 years or longer and three of nine (33%) have lived longer than 1 year. The data of TTP status show that nine of 18 (50%) patients were progression-free for 0.5 years or longer and two of nine (22%) for 1 year or longer. The patients being less than 0.5 and 1 year in the study were neglected in this calculation. In the control group [3], eight of 20 (40%) patients lived 0.5 years or longer and three of 20 (15%) 1 year or longer. Four of 20 (20%) patients had a TTP of 0.5 years or longer. One-year TTP was not observed at all. For the analyses, according to Kaplan–Meier all RP101 dose groups were combined, which included 21 enrolled patients (Fig. 5). The results are similar but not identical to those of the pilot study with GEM + CIS + RP101 (Fig. 4). To date, adverse events are consistent with those observed with GEM or the underlying disease.

Pharmacokinetic results

RP101

All measurements were performed within 9 h. Therefore, only the first three intakes of RP101 tablets were relevant in this context, and, as a consequence, in dose groups IV and V the intake of tablets was identical. When the dose was doubled from 125 to 250 mg, the mean maximum RP101 concentration in plasma increased 5-fold from 290 ± 152 to 1549 ± 1058 ng/ml (Fig. 6).

BVU

The mean maximum BVU concentration increased nearly proportional with the dose of RP101 from 1525 ± 1012 to 4637 ± 1353 ng/ml.

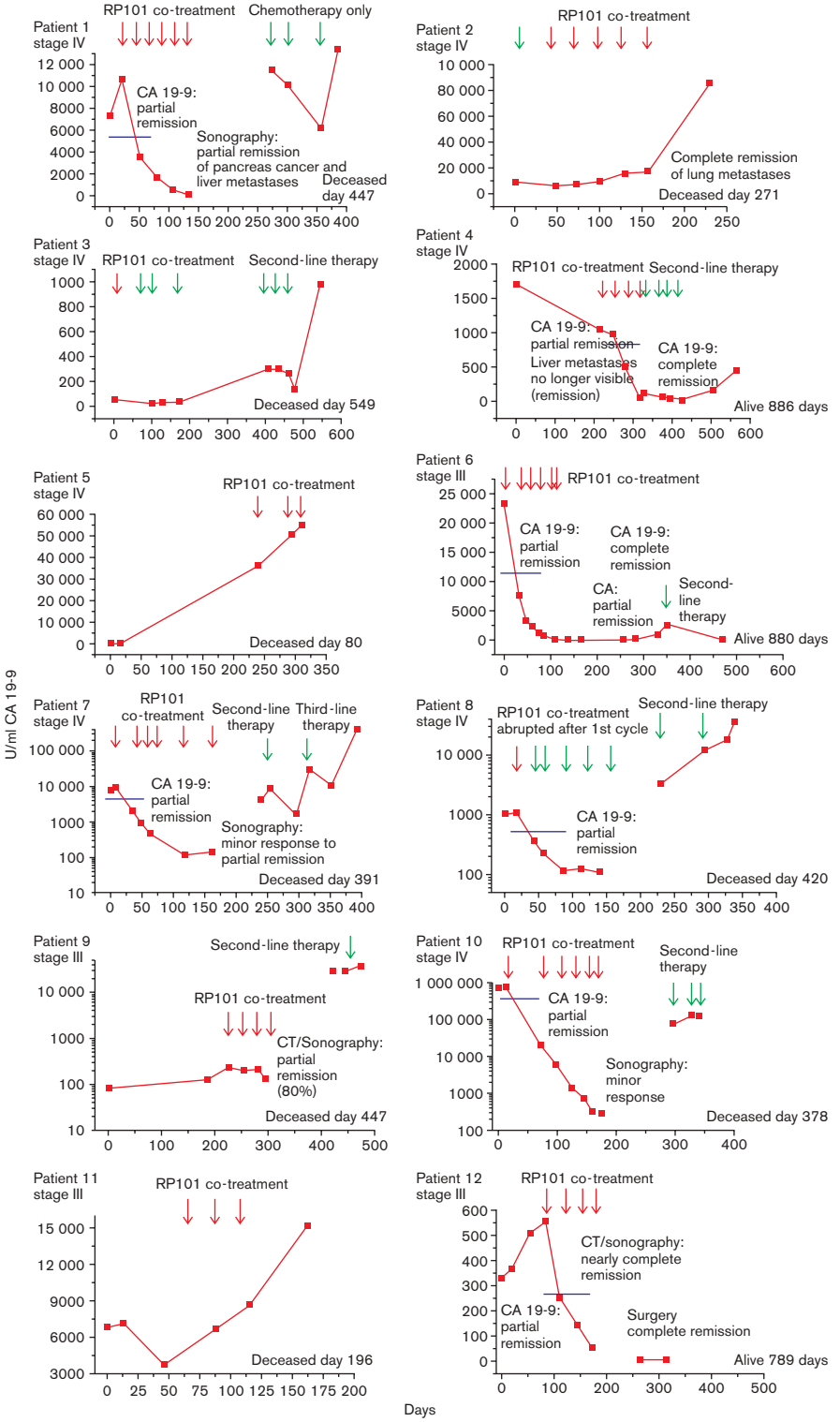
GEM

The maximum concentrations were measured immediately after completion of the 30-min infusion. The maximum values varied between 3185 and 29040 ng/ml. RP101 was given 30 min before GEM and the second time 4 h later. At this time, GEM was not detectable any more. Therefore, an influence on GEM concentration by RP101 could only be exerted by the first intake of tablets. For this reason, only the first two doses of RP101 (125 and 250 mg) can be compared. The GEM maximum values appear to rise with increase of the RP101 dose but the large degree of variability between individual patients should be considered (Fig. 6).

Discussion

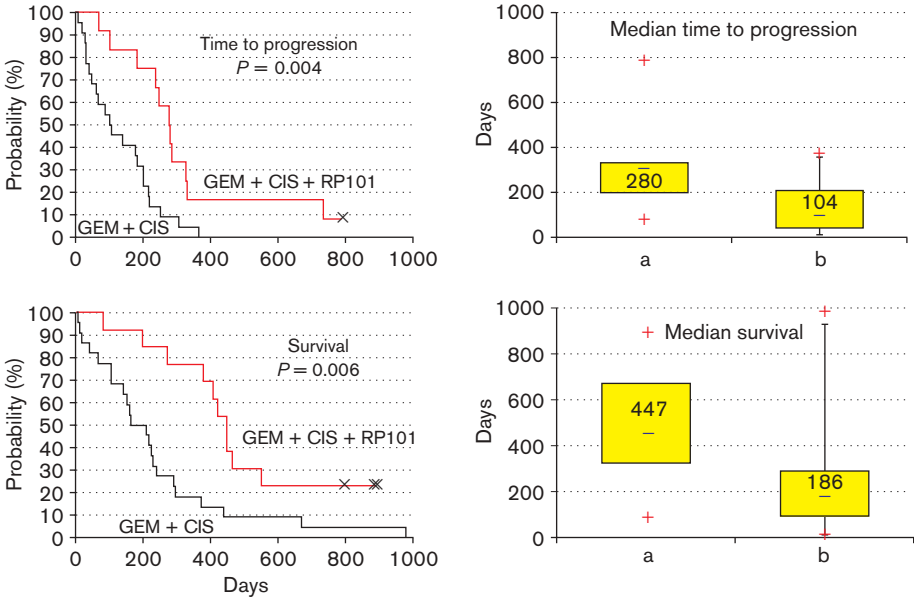
The prognosis of patients with advanced pancreatic cancer is very poor and effective treatments are lacking. In the pivotal study of GEM, median survival was still less than 6 months [7]. Co-treatment with erlotinib, the second approved drug, does not have a clinically significant effect. GEM plus erlotinib treatment vs. GEM plus placebo treatment showed increased overall survival (6.37 months vs. 5.9 months, $P = 0.025$), improved 1-year survival (24 vs. 17%) and improved TTP (3.75 vs. 3.55 months, $P = 0.003$), while skin rash (6

Fig. 3



Pilot study: course of disease followed by tumor marker CA19-9. Red arrows indicate co-treatment RP101 + GEM + CIS; green arrows indicate second-line therapy with different cytostatics alone. RP101, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU); GEM, gemcitabine; CIS, cisplatin.

Fig. 4



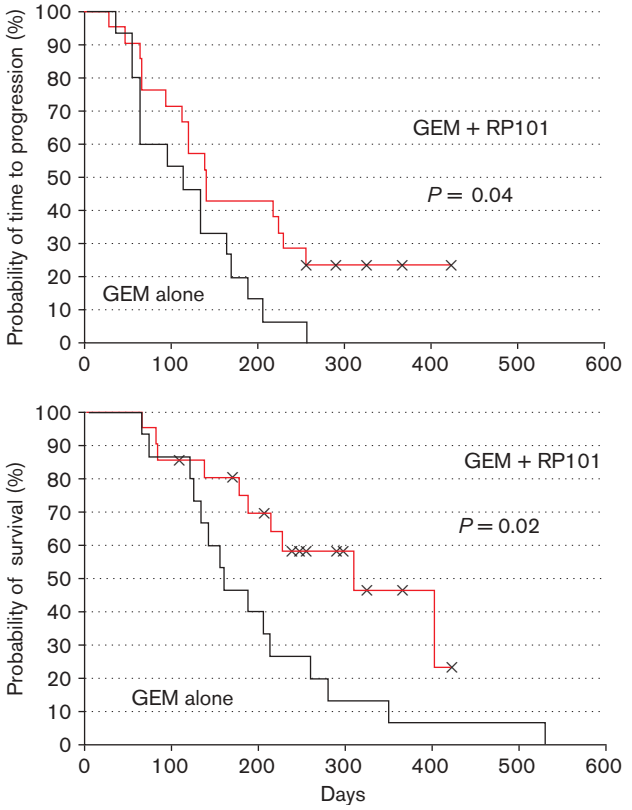
Pilot study: median time to progression or median survival according to box and whisker plots and probability of time to progression or of survival according to Kaplan–Meier. Red line or a=RP101 co-treatment group; black line or b=chemotherapy alone group; X=one patient without progression and three living patients, respectively. RP101, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU).

Table 3 Characteristics of patients treated with GEM + RP101

Patient no.	Age (years)	Stage	Survival (days)	Remissions sonography and CT	TTP (days)	Cycle RP101	CA19-9: highest and lowest level
6.000 mg RP101 per cycle							
101	60	T4NxM1G2	189	SD (4000, 3 × 6000, 2.000 mg RP101)	119	5	902/142
102	68	T3N1MxG3	228	SD	139	6	144/10
103	50	T4N1M0G1	403	SD	140	6	943/256
104	72	T4N1M1G3	178	SD	140	6	12465/7229
7.500 mg RP101 per cycle							
105	41	T3N0M1G2	>424	CR	>424	8	6/3
106	46	T4N0M0G2	214	SD	112	5	98/95
107	73	T3N0M1G3	82	SD (2 × 7500, 2500 mg RP101)	64	3	19/6
108	62	T4NxM1G2	309	SD	217	7	1875/494
201	69	T4N1M1Gx	>366	SD	>366	8+	1640/698
9.000 mg RP101 per cycle							
109	67	T4N1M0Gx	>326	SD (9000, 8500, 4 × 7500 mg RP101)	>326	6	26/18
202	69	T4N1M1G3	>298	SD	255	6	1368/490
110	59	TxNxM1G3	137	SD (3 × 9000, 6000 mg RP101)	94	4	79200/22.1589
111	56	T4N1M0Gx	>290	SD	>290	8+	3721/190
10.500 mg RP101 per cycle							
112	41	T3N0M1Gx	66	SD	66	2	14000/?
301	70	T4N1M1G1	>255	SD (2 × 10500, 10000, 3 × 9000 mg RP101)	>255	6+	556/92
(302)	(64)	T3N0MxG3	excluded: without any progressive disease cycle 2 not completed			[1]	(9564/7458)
203	68	TxN0M1Gx	>248	SD (10500, 9500, 9000, 7500)	229	6	7709/2973
204	62	T2N1M1G3	>240	SD (2 × 10500, 9125, 9000 mg RP101)	223	5	2881/1797
12.000 mg RP101 per cycle							
303	62	T3N1M0Gx	>207	SD (11500, 9500 mg RP101)	46	2	148/357
205	39	T4N0M1G2	>171	SD (12000, 11750, 12000 mg RP101)	119	5	63801/8389
206	41	T2N0M1G3	83	PD	28	2	13/14
207	67	T4N0M1G3	>109	SD	65	2+	218
∅ 21	59	6xIII, 15xIV	>230	5%	>177	5 (2–8)	65% PR

GEM, gemcitabine; CT, computed tomography; TTP, time to progression; SD, stable disease; CR, complete remission; PD, progressive disease; PR, partial remission.

Fig. 5



Dose finding study: probability of time to progression or of survival according to Kaplan–Meier. Red line = RP101 co-treatment group; black line = chemotherapy alone group; X = without progression or living patients, respectively. RP101, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU).

vs. 1%) and diarrhea increased (6 vs. 2%). Our two studies roughly showed the tendency to double the survival time of the controls, and the combination GEM + CIS + RP101 seemed to be more effective than the combination GEM + RP101. In both studies, adverse events were consistent with those observed with GEM, CIS or the underlying disease.

Although there is a large inter-individual degree of variability of the GEM maximum in the present study and in other studies [8], it might be possible that higher concentrations of RP101 may enhance the maximum levels of GEM. Therefore, it cannot be excluded that a further increase of RP101 doses could lead to an enhancement of GEM adverse effects. In the present study, no such effects were observed. Moreover, dose finding is complicated by the unusual metabolism with rapid degradation of RP101 to its ineffective metabolite BVU followed by resynthesis of the original drug from its metabolite following the desoxyribosyl transfer reaction [9]. In this way, the actual half-life of BVDU is significantly increased [10] and this may be one reason for the disproportional increase of RP101 concentration observed.

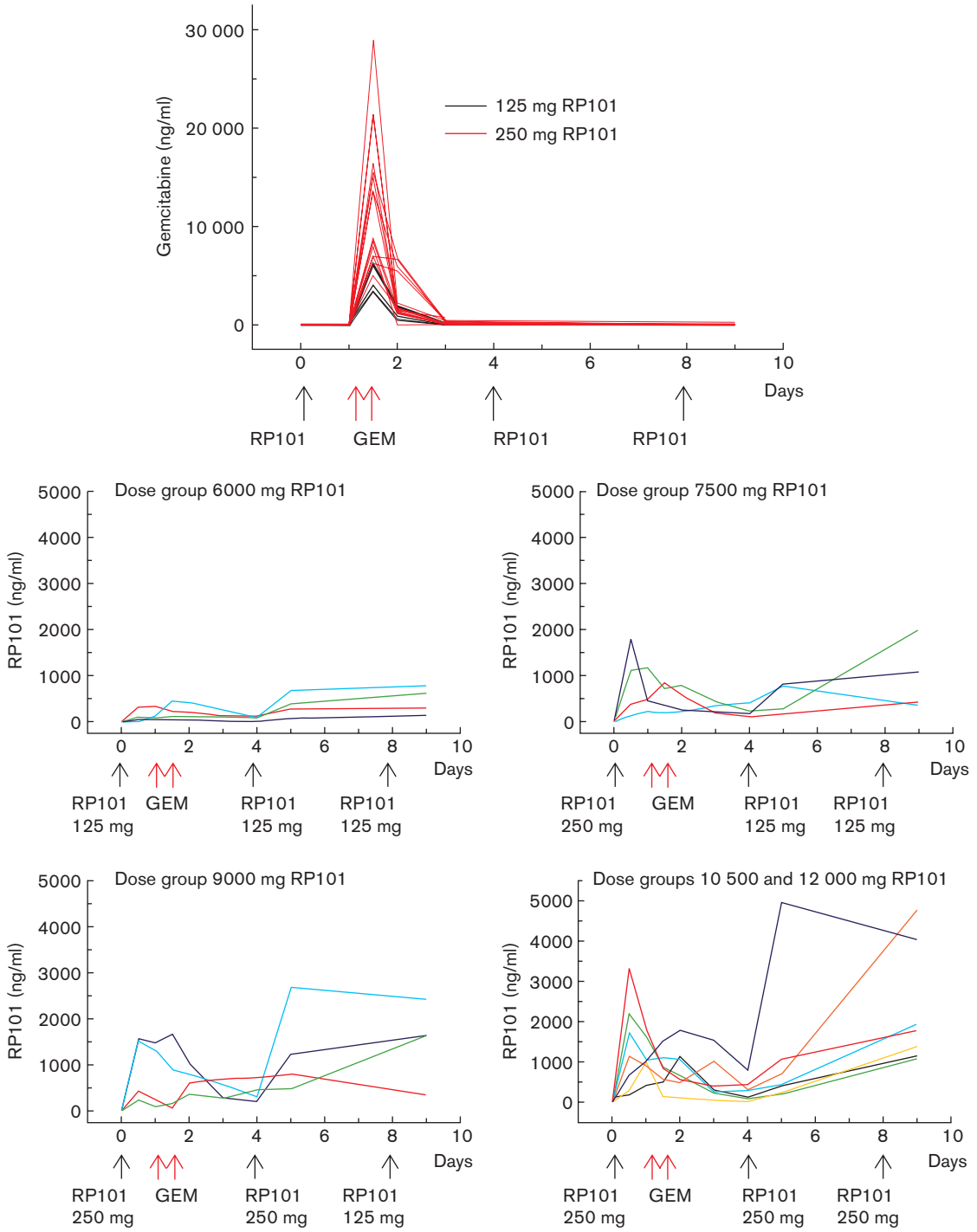
Possible explanations for the efficacy of RP101 to improve chemotherapy can be derived from our observations in cultured pancreatic tumor cells. In general, reasons for insensitivity to chemotherapy and circumvention of drug-induced apoptosis are the altered expression of DNA repair genes, oncogenes, resistance genes and genes relevant for immune responses. These effects were antagonized by RP101. In particular, RP101 downregulated genes involved in the suppression of antitumor activity and apoptosis such as Stat3 and its target VEGF [11], which otherwise promotes tumor angiogenesis [12]. Repression of Stat3 activates dendritic cells and adaptive immunity towards cancer cells by blocking both interleukin-10 and VEGF signaling [13,14], and, therefore, blocking of VEGF could be useful in cancer therapy [15]. Kortylewski *et al.* [16] observed markedly enhanced function of dendritic cells, T cells, NK cells and neutrophils in tumor-bearing mice with Stat3$^{-/-}$ hematopoietic cells, and showed that tumor regression requires immune cells. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity.

Constitutively activated Stat3 is oncogenic [17], contributes to the development of various human cancers [18] and Stat3β gene therapy leads to tumor regression [19]. Thus, Stat3 promotes cell survival and renders cancer cells resistant to chemotherapy, and, conversely, inhibition of Stat3 signaling increases sensitivity to chemotherapeutic agents [17]. In pancreas carcinoma cells, overexpression of Stat3 is leading to suppression of apoptotic responses [13]. In our experiments, overexpression of Stat3 and VEGF, and the phosphorylation of Stat3 were prevented by RP101 co-treatment.

A group of TNF-α-related ligands, LTA, LTB and TNFSF14/LIGHT, were upregulated by RP101 and the combination of RP101 + GEM in the human pancreas carcinoma cell lines CAPAN-2 and AsPC-1. LTA can form soluble homotrimers that are secreted [20] and bind to TNF receptor 1 [21], thereby inducing apoptosis [20]. LTB forms membrane-bound heterotrimers only together with LTA [21], which bind to LTBR [22], inducing caspase-independent cell death through the non-death domain [23–25].

Drayton *et al.* [26] generated transgenic mice using the rat insulin promoter II (RIP) driving overexpression of murine LTA and LTB RIPLTα, and especially RIPLTαβ transgenic mice exhibited extensive infiltrates of T and B cells in the pancreas at the sites of transgene expression, which caused distortion of normal islet architecture. Therefore, pancreas carcinoma cells overexpressing LTA,

Fig. 6



Pharmacokinetic results. GEM: the maximum concentrations were measured immediately after completion of the 30-min infusion. RP101 is given 0.5 h before GEM and the second time 4 h later. GEM disappeared within this time. Therefore, an influence on GEM concentration by RP101 could only be exerted by the first intake of tablets. This leads to the result that only two different doses of RP101 (125 and 250 mg) can be compared. RP101: all measurements are performed within 9 h. This means that only the first three intakes of tablets were relevant. In dose groups IV and V, the intake of tablets was identical. RP101, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU); GEM, gemcitabine.

LTB and LTBR (which is also slightly upregulated by RP101) may modulate immune response or eradicate each other in a paracrine fashion within solid tumors.

We found TNFSF14/LIGHT and ICAM-1 upregulation in pancreas carcinoma cells upon RP101 co-treatment. TNFSF14/LIGHT-induced inhibition of tumor growth is

Table 4 Summary of RP101 effects

Repeated treatment with cytostatic drugs leads to induction of chemoresistance and poor prognosis by	Repeated treatment with cytostatic drugs + RP101 could prevent induction of chemoresistance and poor prognosis by
Overexpression of	Inhibiting overexpression of
● Different survival pathways	● Different survival pathways
● Different DNA repair genes	● DNA repair genes like *UBE2N* and *APEX1*
● Different oncogenes	● Oncogenes like *STAT3*, *DDX1* and *JUN-D*
● Uridine phosphorylase (UPase)	● Uridine phosphorylase (UPase)
Overexpression of Stat3 and its target VEGF, leading to	Inhibiting overexpression of Stat3 and its target VEGF, potentially leading to
● Blockade of the initiation of anti-tumor immunity	● Activation of anti-tumor immunity by overexpression of lymphotoxins α and β, natural killer cell transcript 4 (NK4), tumor necrosis factor LIGHT/TNFSF-14, ICAM-1
● Enhancement of tumor cell proliferation	● Inhibition of tumor cell proliferation
● Prevention of apoptosis	● Maintenance of apoptosis
Downregulation of	Upregulation of
● DT-diaphorase	● DT-diaphorase
● Caspases	● Caspase 3
All effects together cause suppression of apoptosis and induction of chemo-resistance	All effects together cause induction of apoptosis and maintenance of chemosensitivity
Suppression of apoptosis leads to	Maintenance of apoptosis leads to
● Genomic instability by recombination and other events	● Suppression of induced recombination
● Amplification of resistance genes like *MDR* and *DHFR*	● Suppression of amplification of resistance genes like *MDR* and *DHFR*

highly correlated with the upregulation of ICAM-1 [27], which is a ligand that activates NK cells. TNFSF14/LIGHT also binds to LTBR and induces caspase-dependent and independent death in tumor cells expressing LTBR and/or herpes virus entry mediator [28,29]. Both receptor types are expressed in CAPAN-2 and AsPC-1 cells. Furthermore, NK4, which was recently shown to upregulate TNF-α production in macrophages when secreted [30], was overexpressed upon RP101 co-treatment.

Therefore, the effects of RP101 were likely due to the downregulation of Stat3 and its target VEGF, and to the increase in antitumor immunity that is reflected by enhanced expression of LTA and LTB, tumor necrosis factor LIGHT/TNFSF14, and NK4 (interleukin-32). These gene products influence apoptosis [18,27,31,32].

In cultured human pancreatic tumor cells, RP101 inhibited gene products involved in DNA repair such as APEX1, which was also a main effect observed in other tumor cell lines [1]. Treatment with GEM or other cytostatic drugs leads to apurinic sites, which triggers DNA repair, including the induction of APEX1 to restore DNA replication and genetic integrity. Thus, inhibition of DNA repair genes like APEX1 during anticancer treatment increases chemosensitivity, because it was shown that silencing of APEX1 expression by RNA interference enhanced DNA nicking and nearly doubled specific cell lysis [27].

Finally, RP101 downregulated UPase expression in BxPC-3 and AsPC-1 human pancreas carcinoma cell lines. It was demonstrated that UPase is highly expressed in a panel of pancreas cancer cell lines and that UPase can potentially be useful in tumor targeting and as a tumor marker [33]. In squamous cell carcinoma, high staining of UPase in primary tumors was frequently associated with the presence of lymph node metastases and lower overall survival [34]. Breast carcinoma patients with high UPase levels had a worse prognosis than those with low levels [34].

As a result of the multiplicity of mechanisms of action it is difficult to understand which mechanism or mechanisms are responsible for the therapeutic effect of the RP101 combination chemotherapy. The numerous immunomodulatory and chemosensitizing properties of RP101 are probably working in concert to optimize chemotherapy and to inhibit chemoresistance. Table 4 shows the main effects of RP101 in relation to each other to allow a better understanding of its mode of action [1,35–38].

The present results prompted us to start a phase II/III study with GEM in combination with RP101. As the effectivity of RP101 is independent of the cytostatic drug used or the tumor entity used, corresponding clinical studies are scheduled.

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