

The combination of everolimus and zoledronic acid increase the efficacy of gemcitabine in a mouse model of pancreatic adenocarcinoma

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ABSTRACT

Background: Gemcitabine is a standard treatment for pancreatic adenocarcinoma. Many mechanisms are involved in gemcitabine resistance, such as reduced expression of the human equilibrative nucleoside transporter 1 (hENT1) membrane transporter, deoxycytidine kinase deficiency, and changes in the signal transmission of mitogen-activity protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) pathways.

Aim: To evaluate the anti-tumor efficiency of blocking signaling pathways using combined action of gemcitabine, everolimus and zoledronic acid versus gemcitabine alone in a mouse subcutaneous xenograft.

Methods: Implantations of two human pancreatic adenocarcinoma cells lines (PANC1, K-ras mutated and gemcitabine-resistant; and BxPc3, wild-type K-ras and gemcitabine-sensitive) were performed on male athymic nude mice. The mice received different treatments: gemcitabine, gemcitabine plus everolimus, everolimus, gemcitabine plus zoledronic acid, everolimus plus zoledronic acid, or gemcitabine plus everolimus and zoledronic acid, for 28 days. We measured the tumor volume and researched the expression of the biomarkers involved in the signaling pathways or in gemcitabine resistance.

Results: In wild-type K-ras tumors, the combinations of gemcitabine plus everolimus; zoledronic acid plus everolimus; and gemcitabine plus zoledronic acid and everolimus slowed tumor growth, probably due to caspase-3 overexpression and reduced Annexin II expression. In mutated K-ras tumors, gemcitabine plus everolimus and zoledronic acid, and the combination of zoledronic acid and everolimus, decreased tumor volume as compared to gemcitabine alone, inhibiting the ERK feedback loop induced by everolimus.

Conclusion: The combination of zoledronic acid and everolimus has an antitumor effect and could increase gemcitabine efficacy.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is becoming the second leading cause of cancer-related

deaths in France [1], and fourth in the world [2]. Moreover, it is the digestive cancer with the worst prognosis, with a 5-year overall survival rate of 7%, as found in the EURO CARE study [3]. Gemcitabine

Table 1: Groups of treatment

BxPc3 n=59	P n=8	G n=9	G + E +AZ n=10	E+AZ n=10	G+AZ n=7	G+E n=8	E n=7
PANC-1 n=26	P n=8	G n=5	G + E + AZ n=7	E + AZ n=6			

Mice were separated depending on Xenograft of cells lines BxPc3 and PANC-1 and they received different combination of treatments.

Placebo (P), Gemcitabin (G), Gemcitabin + Everolimus + Zoledronic acid (G+E+AZ), Gemcitabin + Everolimus (G+E), Gemcitabin + Zoledronic acid (G + AZ), Everolimus + Zoledronic acid (E+AZ) and Everolimus (E).

(2'-2'-difluorodeoxycytidine) is a standard chemotherapy treatment for all stages of pancreatic adenocarcinoma. However, the survival benefit and clinical impact remain modest due to the high degree of intrinsic and acquired resistance. In the last decade, many randomized trials involving gemcitabine combinations were performed on metastatic patients, however they failed to demonstrate a statistically significant survival advantage over gemcitabine alone, with the exception of combinations with erlotinib, which provided few benefits [4], and more recently with nab-paclitaxel [5]. However, the interest of using gemcitabine in the treatment of PDAC is still relevant. Theoretically, it remains the standard treatment for patients over the age of 75 and patients whose WHO status is less than or equal to 2. In an adjuvant situation, it currently represents the reference treatment in combination with capecitabine [6]. Gemcitabine is a pyrimidine analog transformed by deoxycytidine kinase (dCK) into gemcitabine diphosphate (dFdCTP) and gemcitabine triphosphate (dFdCTP). DNA incorporation of these metabolites inhibits DNA synthesis and induces apoptosis. The induction of apoptosis through caspase signaling is an important action mechanism [7]. Gemcitabine resistance mechanisms are complex, involving both the tumor microenvironment and the intrinsic resistance [8]. Gemcitabine resistance seems to be partly related to changes in gene expression involved in gemcitabine transport and metabolism. Two main types of resistance were described: reduced expression of human equilibrative nucleoside transporter-1 (hENT-1) and deficiency in dCK activity, which plays a major role in the cellular transformation of gemcitabine into active metabolites [8]. Other mechanisms are also involved in gemcitabine resistance, such as Annexin a2 overexpression through activation of the Akt/mTOR apoptotic pathway [8, 9]. It has been shown that everolimus has an additive antiproliferative effect in gemcitabine-treated pancreatic tumor cells *in vitro* [10]. Otherwise, PDAC is the cancer with the highest K-ras mutation frequency (>90%) [11]. The MAPK pathway,

particularly K-ras activating mutations, plays a major role in pancreatic carcinogenesis [12]. Ras proteins alternate between GTP-bound that represents the “On state” and GDP-bound that was the “Off state”. The transition between these two states requires proteins accelerating GTP hydrolysis, GTPase activating proteins (GAPs). Mutated K-ras prevents the intrinsic and GAPs catalyzed hydrolysis of GTP, thereby generating permanently active RAS and constitutive activation of cell proliferative signals [13]. This mutation leads to a reaction cascade marked by the successive activation of proteins involved in the MAPK pathway: Raf, MEK and ERK. It has also been suggested that pancreatic carcinogenesis could strongly rely on the dysregulated activity of the p21ras. Indeed, it has been shown that zoledronic acid has antiproliferative effects on the p21ras/Raf1/MEK/ERK1-2 mitogenic pathway and Pkb/Akt survival signaling through ERK inhibition. Zoledronic acid also induces apoptotic death of human pancreatic cancer cells *in vitro* [14]. Conversely, it has been shown that K-Ras mutation induced feedback ERK activation contributes to the rapalog resistance in pancreatic ductal adenocarcinomas [15] and that PI3K pathway activation mediates resistance to MEK inhibitors in K-Ras mutant cancers [16]. It is therefore not surprising that everolimus used as a single agent does not have an anti-tumor effect in patients with gemcitabine-refractory metastatic PDAC [17]. We hypothesized that simultaneous blocking of both signaling pathways using everolimus and zoledronic acid could increase the efficacy of gemcitabine.

RESULTS

Eighty-five mice were included in the study, divided according to Table 1.

The initial tumor volume was 113.1 +/-24.4 mm³ in the BxPc3 group, compared to 108.9 +/-20.7 mm³ in the PANC1 group. There was no significant difference between groups according to the non-parametric Mann-Whitney-Wilcoxon test (p=0.60). Moreover, there was no

significant difference between the weights of the mice in the different groups (Mann-Whitney-Wilcoxon, $p=0,06$).

Weight variation

The weight variation was no different between the BxPc3 and PANC-1 groups.

Tumor volume

The mean change in tumor volume was $450.9 \pm 189.3 \text{ mm}^3$ for the placebo, $265.1 \pm 126.9 \text{ mm}^3$ for gemcitabine, 76.1 ± 56.2 for G+E+AZ and 78.0 ± 52.8 for E+AZ in BxPc3 group.

The mean change in tumor volume was $123.9 \pm 124.3 \text{ mm}^3$ for the placebo, $75.0 \pm 23.1 \text{ mm}^3$ for gemcitabine, -40.4 ± 34.8 for G+E+AZ and -44.8 ± 19.4 for E+AZ in PANC-1 group. The tumor volume change was significantly higher in BxPc3 than in PANC-1: placebo $p=0.011$; gemcitabine $p=0.041$; E+AZ $p=0.006$ and G+E+AZ $p=0.006$.

In the placebo and gemcitabine groups, the tumor volume increased, however this increase was smaller in the PANC-1 group. For E+AZ and G+E+AZ, the tumor

volume increased slightly in the BxPc3 group, whereas it decreased in the PANC-1 group. These differences were always significant after weight variation adjustment.

BxPc3 group

The tumor volume variation was significantly higher in the placebo group versus gemcitabine ($p=0.032$) G+E+AZ ($p=0.006$), G+E ($p=0.005$), G+AZ ($p=0.021$) versus E+AZ ($p=0.005$) and E ($p=0.011$). This increase was significantly lower for G+E+AZ ($p=0.011$), G+E ($p=0.012$) and E+AZ ($p=0.011$) as compared to the gemcitabine group. The best tumor response was observed in G+E (Figure 1).

PANC1 group

There was no significant tumor volume variation between the placebo and gemcitabine groups ($p=0.721$). These variations significantly decreased for G+E+AZ ($p<0,01$) and E+AZ ($p<0,01$) as compared to placebo and gemcitabine ($p=0.019$ for G+E+AZ and $p=0.024$ for E+AZ) (Figure 2).

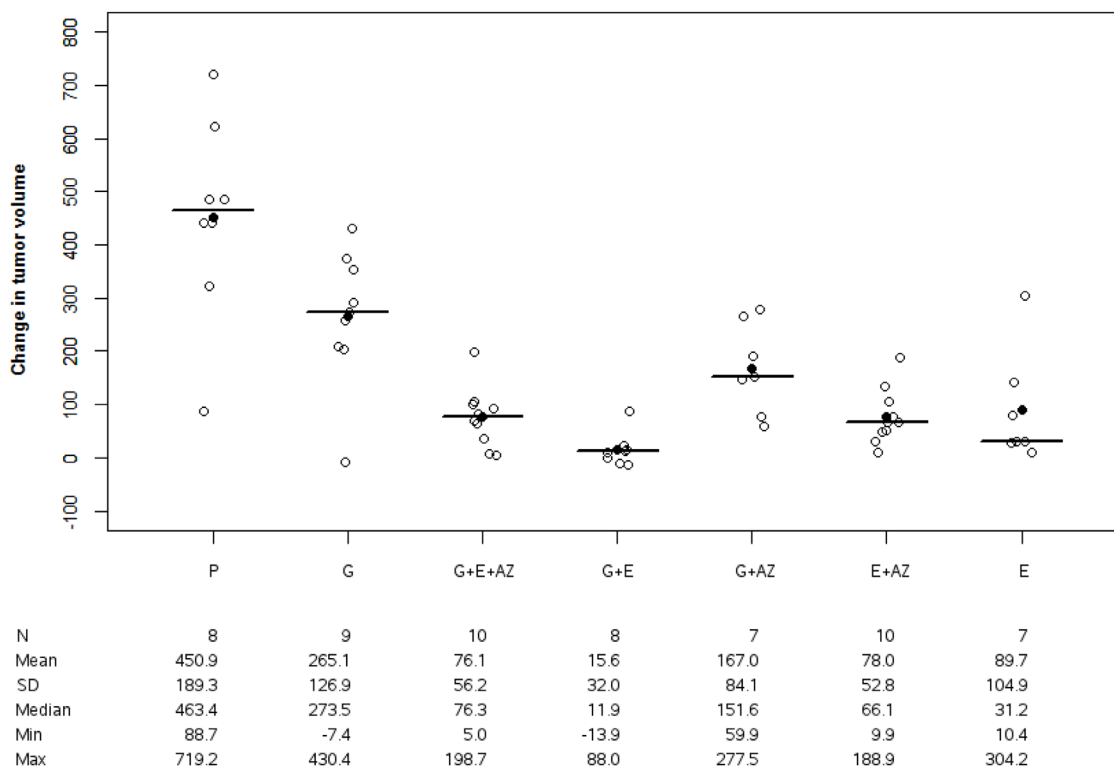


Figure 1: Tumor volume change in BxPc3. Tumor volume change was significantly decreased with G+E+AZ, G+E in BxPc3 mices. Placebo (P), Gemcitabin (G), Gemcitabin + Everolimus + Zoledronic acid (G+E+AZ), Gemcitabin + Everolimus (G+E), Gemcitabin + Zoledronic acid (G + AZ), Everolimus + Zoledronic acid (E+AZ) and Everolimus (E). SD: standard deviation.

Signaling pathways

Ras/Raf/MAPK

BxPc3 versus PANC-1 group

In BxPc3, the ERK-p expression was 86.9 +/-31.2% in placebo, 120.3 +/- 27.6 % in gemcitabine, 160.2 +/- 55.4% in E + AZ and 134.3 +/- 48.5% in G + E + AZ. In PANC-1, the ERK-p expression was 111.8 +/- 65.9 in placebo, 54.0 +/- 7.3 % in gemcitabine, 142.4 +/-56.0 in E + AZ and 69.8 +/- 15.1 in G + E + AZ. There was an higher expression of ERK-p in gemcitabine BxPc3 group than in gemcitabine PANC-1 group (p=0.008) and in G+ E+ AZ in BxPc3 versus in G + E + AZ in PANC-1 (p=0.043).

BxPc3 group

There was no significant difference in Raf1 tumor expression between groups (p=0.112) (Table 2).

There was an overexpression of ERK-p for E (p=0.028), G+E (p=0.003), E+AZ (p=0.009), G + AZ (p=0.037) and G + E + AZ (p=0.026) as compared to the placebo (Figure 3). And there was an overexpression of ERK-p for G+ E as compared to gemcitabine (p=0.016).

There was no significant difference in Ki67 tumor expression between groups (p=0.140).

PANC-1 group

Only ERK-p tumor expression was studied.

There was no significant difference in groups as compared to the placebo. The ERK-p tumor expression significantly increased for E+AZ as compared to gemcitabine (p=0.011) (Figure 4).

PI3K/AKT/MTOR

BxPc3 versus PANC-1 group

There was a lower expression of AKT in placebo BxPc3 versus placebo PANC-1 (p=0.016) (Table 2). There was no difference between the others groups.

BxPc3 group

There was no significant difference in tumor expression of AKT between groups (p=0.209) (Table 2).

There was no significant difference in tumor expression of mTOR between groups (p=0.90) (Table 2).

There was an overexpression of Caspase-3 tumor in G+ E (p= 0.019), G + AZ (p= 0.003) and E (p= 0.007) versus placebo (Figure 5).

PANC-1 group

There was no significant difference in tumor expression of AKT between groups (p=0. 057) (Table 3).

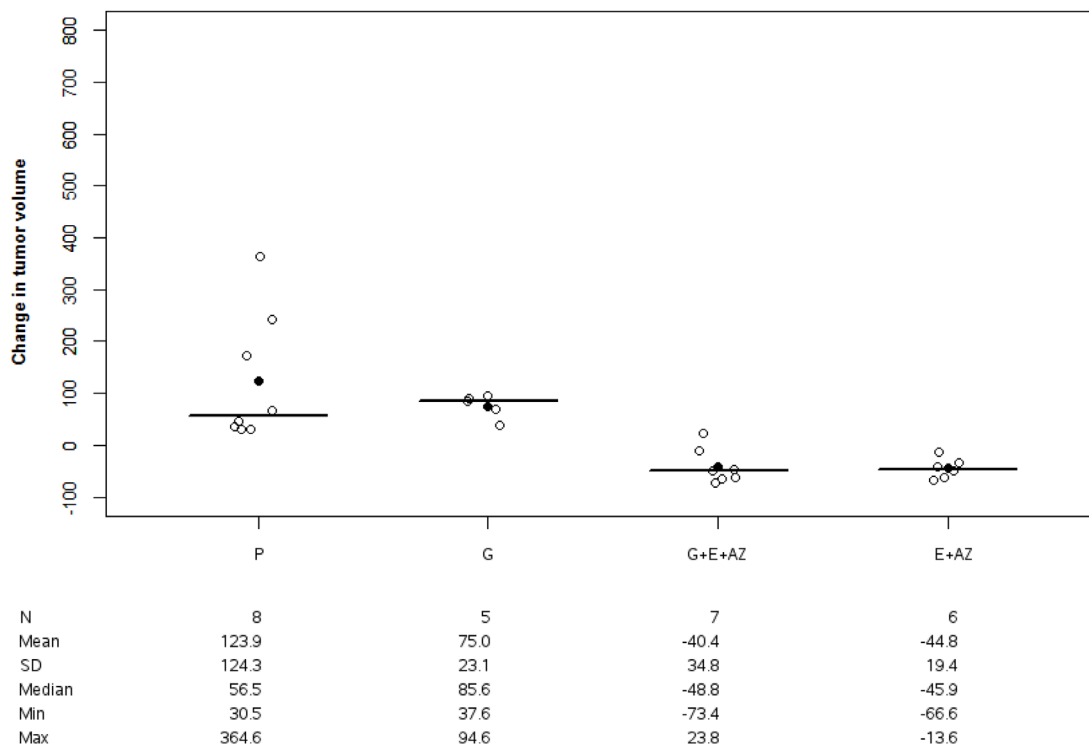


Figure 2: Tumor volume change in PANC1. Tumor volume change was significantly decreased with G+E+AZ and E+ AZ in PANC-1 mice. Placebo (P), Gemcitabine (G), Gemcitabine + Everolimus + Zoledronic acid (G+E+AZ) and Everolimus + Zoledronic acid (E + AZ). SD: standard deviation.

Table 2: ERK-p, RAF-1, AKT, mTOR, Ki67, Annexin II, Caspase 3, hENT1 expression in BxPC3

n=59	P n=8	G n=9	G + E + AZ n=10	E + AZ n=10	G + AZ n=7	G + E n=8	E n=7	p
ERK-p								<0.018
n	8	7	10	9	7	7	5	
Me +/-SD	86.9 +/-31.2	120.3 +/-27.6	134.3 +/- 48.5	160.2 +/-55.4	143.4 +/-68.3	172.0 +/- 42.7	134.5 +/- 29.6	
Median	77.1	122.9	130.3	142.6	140.7	160.3	146.0	
Min	55.85	74.86	70.02	76.99	67.42	124.64	98.08	
Max	147.56	153.53	226.90	250.56	283.04	235.76	165.52	
RAF-1								0.112
n	6	9	6	6	7	8	7	
Mean +/- SD	99.7 +/-34.9	79.4 +/-27.5	97.3 +/- 40.4	130.1 +/- 38.9	120.7 +/- 24.3	124.8 +/-38.2	110.2 +/-39.7	
Median	81.6	70.9	106.5	130.3	117.3	120.2	92.6	
Min	72.59	44.93	39.21	70.73	95.33	68.4	61.8	
Max	148.36	129.7	143.40	184.97	168.47	176.2	170.8	
AKT								0.209
n	8	8	10	9				
Mean +/- SD	81.9 +/- 15.0	87.8 +/- 16.5	89.4 +/- 19.4	108.4 +/- 30.0				
Median	81.2	86.6	91.4	103.0				
Min	61.87	68.17	52.65	70.92				
Max	102.53	113.54	129.03	154.94				
mTOR								0.090
n	7	8	8	6	4	7	7	
Mean +/- SD	86.6 +/- 26.5	116.0 +/- 71.3	75.5 +/- 36.3	57.6 +/- 40.1	124.3 +/- 42.0	91.4 +/- 43.3	123.6 +/-41.5	
Median	86.6	117.8	69.0	48.9	120.2	75.1	116.3	
Min	45.53	10.20	21.33	19.13	84.95	46.21	56.24	
Max	133.13	237.14	148.08	129.19	171.92	172.94	193.18	
Ki67								0.140
n	8	8	10	7	6	7	6	
Mean +/- SD	49.0 +/- 9.0	41.9 +/- 13.6	46.6 +/- 8.4	55.3 +/- 7.9	53.2 +/- 11.4	53.9 +/- 9.3	55.7 +/- 7.2	
Median	48.3	46.6	47.3	51.5	58.3	51.9	55.5	
Min	32.15	20.07	35.81	48.85	34.09	43.29	48.38	
Max	60.69	62.10	59.91	68.80	62.92	66.58	65.23	
Annexin II								<0.001
n	8	8	8	8	4	7	6	
Mean +/- SD	216.3 +/- 135.4	340.9 +/-53.5	168.5 +/- 29.6	221.6 +/- 41.6	78.0 +/- 22.8	107.0 +/- 36.7	146.7 +/- 98.7	
Median	169.2	334.6	183.7	208.0	81.6	119.9	146.4	
Min	100.95	251.52	115.09	178.88	50.44	50.37	32.73	
Max	428.34	424.04	200.66	297.35	98.46	148.47	275.40	
Caspase 3								0.023
n	7	7	7	8	7	5	6	
Mean +/- SD	69.8 +/- 27.3	87.6 +/- 64.4	89.3 +/- 34.7	93.0 +/- 68.2	138.9 +/-28.9	136.9 +/- 47.9	151.6 +/- 57.0	
Median	72.6	69.2	89.0	98.4	125.5	123.6	136.0	
Min	40.40	-2.65	40.26	0.13	106.4	86.76	89.80	
Max	109.98	175.39	129.69	215.15	178.4	216.14	234.64	
hENT1								<0.001
n	8	8	8	7	7	8	7	
Mean +/- SD	140.6 +/- 40.2	167.3 +/- 59.8	177.3 +/- 68.6	198.2 +/- 67.5	116.3 +/-36.4	112.9 +/-24.3	85.1 +/-20.8	
Median	133.6	153.8	168.1	200.2	105.8	111.9	93.9	
Min	83.68	106.19	107.81	109.78	81.7	83.8	40.63	
Max	192.98	306.22	334.14	297.35	170.8	145.8	100.16	

Placebo (P), Gemcitabin (G), Gemcitabin + Everolimus + Zoledronic acid (G+E+AZ) and Everolimus + Zoledronic acid (E + AZ).
SD: standard deviation.

Mechanisms of resistance to gemcitabine

The hENT1 expression in placebo groups was 140.6 +/- 40.2 % in BxPc3 and 79.6 +/- 22.1 % in PANC-1. In gemcitabine groups, this expression was 167.3 +/- 59.8% in BxPc3 and 87.7 +/- 13.4% in PANC-1. The hENT1 expression in E + AZ was 198.2 +/-675% in BxPc3 and 117.7 +/- 29.5% in PANC-1. In G + E +AZ this expression was 177.3 +/- 68.6% in BxPc3 and 126.6 +/-33.6% in PANC-1. There was a significant decrease in hENT1 expression for the placebo (p=0.06), gemcitabine (p=0.007) and E + AZ (p=0.032) in the PANC-1 group as compared to BxPc3. These differences were not significant for G+E+AZ (p=0.126).

In BxPC3, the Annexin II expression was 216.3 +/-135.4% in placebo, 340.9 +/-53.5 % in gemcitabine, 221.6 +/-41.6% in E + AZ and 168.5 +/-29.6 % in G + E + AZ. In PANC-1, The Annexin II expression was 91.4 +/-40.9% in placebo, 119.6 +/- 13.8% in gemcitabine, 96.2 +/- 36.2% in E + AZ and 81.7 +/- 10.4% in G + E +AZ. There was a significant lower in Annexin II expression in placebo (p=0.036), gemcitabine (p=0.003), E+AZ

(p=0.002) and G+E+AZ (p=0.007) in PANC-1 group as compared to BxPc3.

BxPc3 group

There was a significant decrease in tumor expression of hENT1 in everolimus versus placebo (p=0.008, Table 2). And there was a significant decrease of hENT1 in E (p=0.001) and G+ E (p=0.006) versus gemcitabine (data not shown).

Annexin II expression was significantly lower for G + AZ versus placebo (p=0.007) and in G+E+AZ (p=0.001), G+E (p=0.001), G+AZ (p=0.007), E+AZ (p=0.002) and E (p=0.003) as compared to the gemcitabine group (Figure 6).

PANC-1 group

HENT1 expression was significantly higher for G+E+AZ (p=0.017) and E + AZ (p=0.039) as compared to the placebo (Figure 7). There was no significant difference between G+E+AZ (p=0.083) and E+AZ (p=0.136) as compared to the gemcitabine group.

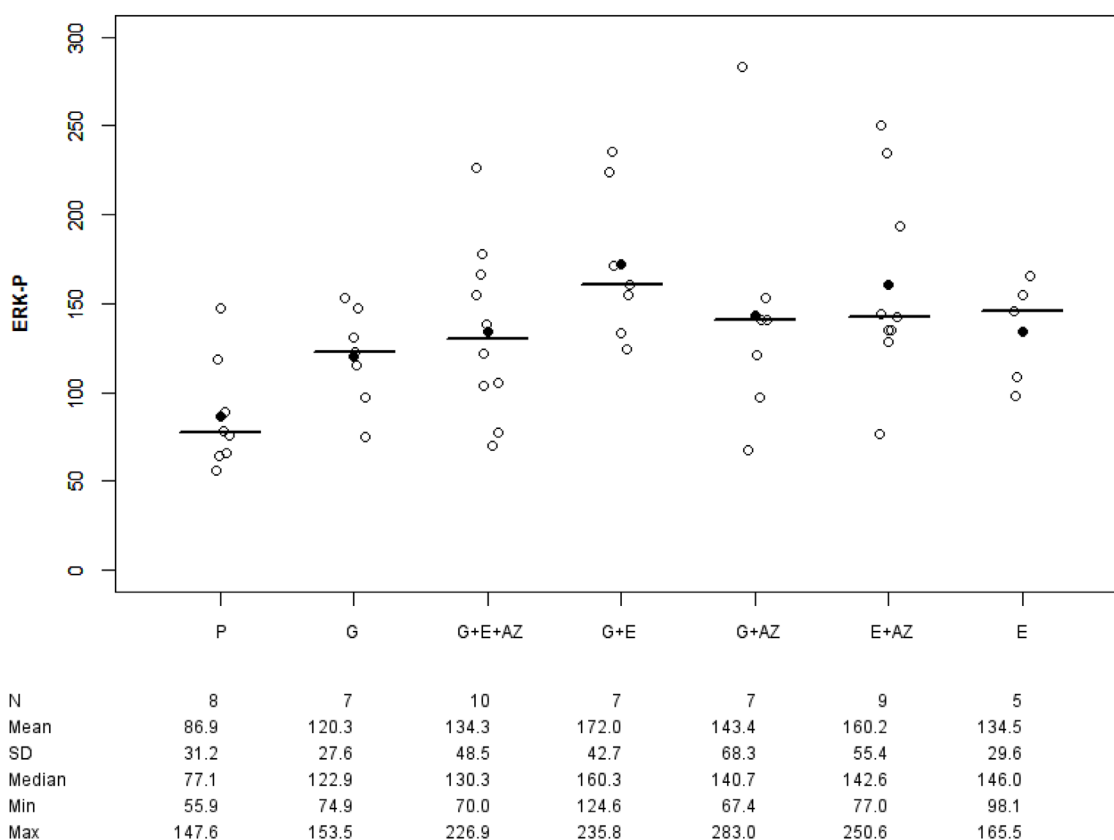


Figure 3: ERK-p expression in BxPc3. Placebo (P), Gemcitabine (G), Gemcitabine + Everolimus + Zoledronic acid (G+E+AZ), Gemcitabine + Everolimus (G+E), Everolimus + Zoledronic acid (E+AZ) and Everolimus (E). SD: standard deviation.

There was no significant difference in tumor expression of Annexin II between the groups ($p=0.261$).

Tolerance

All mice were alive at the end of the study.

For BxPc3, there was a significant reduction in weight between G+E ($p=0.017$) and G+E+AZ ($p=0.031$) as compared to the placebo group, and there was a significant reduction in weight between G+E ($p=0.017$) and G+E+AZ ($p=0.013$) as compared to the everolimus group (data not shown). There was a significant reduction of weight for G+E ($p=0.036$) as compared to the gemcitabine group.

For PANC-1 there was no significant variation in weight of mice.

DISCUSSION

Gemcitabine has been shown to demonstrate significant clinical activity against pancreatic adenocarcinoma [18]. It is therefore important to effectively target patients who respond to gemcitabine and to identify biomarkers that may interfere with its metabolism in order to increase its anti-tumor efficacy. Our study focused on blocking the signaling pathways involved in pancreatic

adenocarcinoma. Our hypothesis was that simultaneous blocking of the mTOR and MAPK pathways upstream ERK pathway could increase gemcitabine's anti-tumor effect. mTOR exists in two distinct functional complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The mTORC1 pathway is also activated by the frequent K-ras mutation in pancreatic cancer and drives cancer progression [19]. It is regulated through two negative feedback loops, which activate PI3K and ERK in pancreatic cancer, resulting in the cancer's resistance to the treatment [20–22]. To test our hypothesis, we used a model of orthotopic xenograft in nude mice from human tumor cell lines, either gemcitabine-sensitive (Wt K-ras, BxPc3 mice) or gemcitabine-resistant (mt-K-ras PANC-1 mice). We chose to block the Akt/mTOR pathway at the mTORC1 receptor using everolimus and to block the MAPK pathway at p21 ras using zoledronic acid, as previously suggested [14]. Almost 90% of pancreatic cancers are K-ras mutated [11]. We had first tested our treatments in wild-type K-RAS cells, sensitive gemcitabine (BxPc3 arm) to select the more efficient combined associations. In our study the associations of gemcitabine plus everolimus, everolimus plus zoledronic acid and gemcitabine plus everolimus and zoledronic acid were more efficient than gemcitabine alone. In the literature, studies have suggested

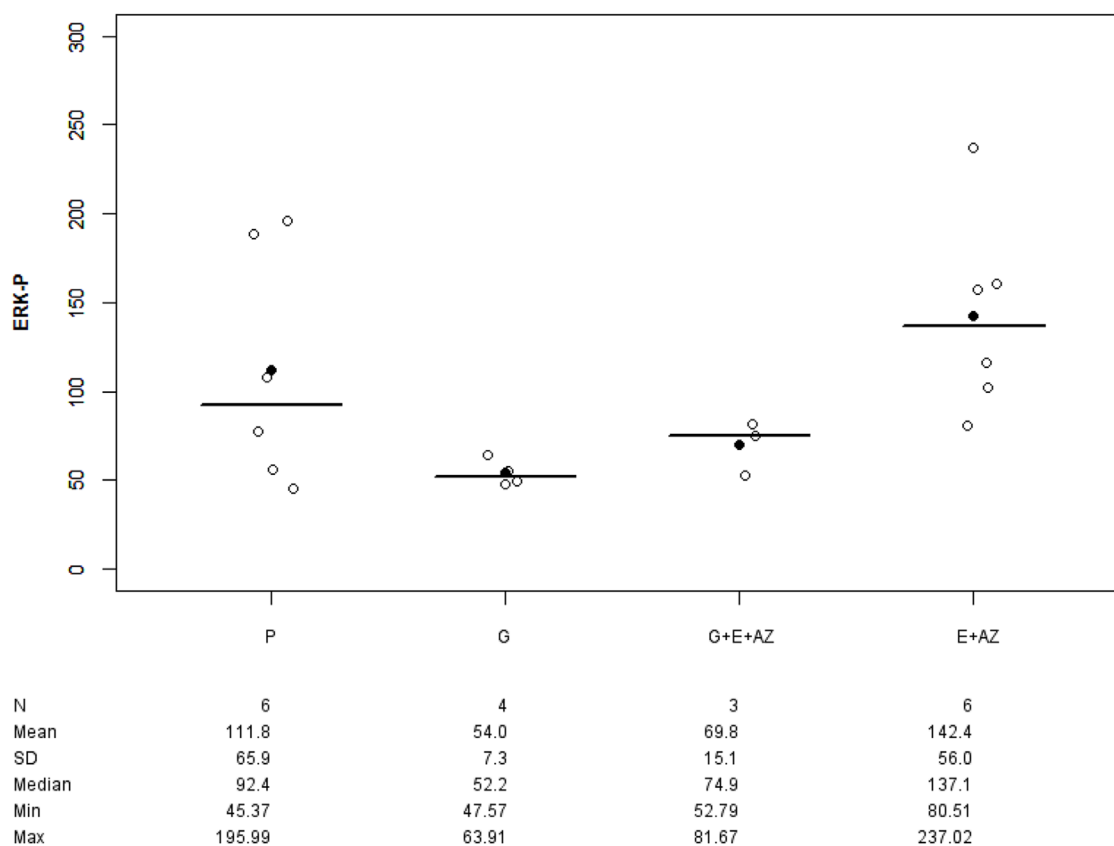


Figure 4: ERK-p expression in PANC-1. Placebo (P), Gemcitabine (G), Gemcitabine + Everolimus + Zoledronic acid (G+E+AZ), Everolimus + Zoledronic acid (E+AZ). SD: standard deviation.

that everolimus used alone inhibits the tumor growth of BxPc3 (wt RAS) cells, but could not block tumor growth in PANC1 (mutated K-ras) mice because everolimus causes an ERK feedback loop and is therefore opposed to the anti-tumor action [15]. So for PANC1 only combinations with everolimus and zoledronic acid were studied. In our study, tumor volume and weight of mice were similar at the initial time of the experimentation, regardless of the group of mice. No toxicity of the different combinations was observed in previous analyses, especially renal failure (data not shown). As in BxPc3 group, the combination of everolimus, zoledronic acid and gemcitabine or zoledronic acid and everolimus are more efficient than gemcitabine alone in mt K-ras tumors. Interestingly, tumor growth decreased in gemcitabine-resistant tumors, whereas it only slowed in gemcitabine-sensitive tumors. In sensitivity tumors, growth is slowed down by gemcitabine alone and this effect is accentuated by the addition of everolimus but not by the addition of zoledronic acid whereas in mutated tumors, the addition of zoledronic acid to everolimus decreases tumor growth. The addition of zoledronic acid could play a role through its interaction with p21ras/raf-1/MEK1/ERK [14].

The mechanisms implied in response of treatments seem to be different depending on cells lines (BxPc3

and PANC-1). Interestingly, it has been suggested that the everolimus-induced PI3K feedback loop does not contribute directly to the rapalog resistance in K-ras-mutated PDAC cells [15]. In our study, an overexpression of ERK-p was found for G+E in BxPc3 and for E+AZ in PANC-1, confirming that everolimus activate ERK without impact on efficacy of treatment. The predominant anti-tumor mechanism in gemcitabine-sensitive tumors appears to be associated with apoptosis, as suggested by caspase-3 overexpression. Constitutive activation of nuclear factor κ B (NF- κ B) is frequently observed in PDAC [23], which suggests that for mt K-ras tumors, zoledronic acid could reduce mTOR resistance and thus enhance the therapeutic efficacy of everolimus, perhaps by inhibiting NF- κ B, as previously shown in breast tumors [24]. Otherwise, in addition, a recent study reported disappointing results of a double inhibition of PI3K and MEK in a second-line treatment following the failure of gemcitabine [25]. This seems to reinforce the idea that the blocking of passing bridges between the two signaling pathways plays a key role. Considered together, these results indicate the zoledronic acid in association with gemcitabine could block the everolimus-induced ERK feedback loop and thus enhance the therapeutic efficacy of everolimus in treating mt K-ras PDAC and could sensitize

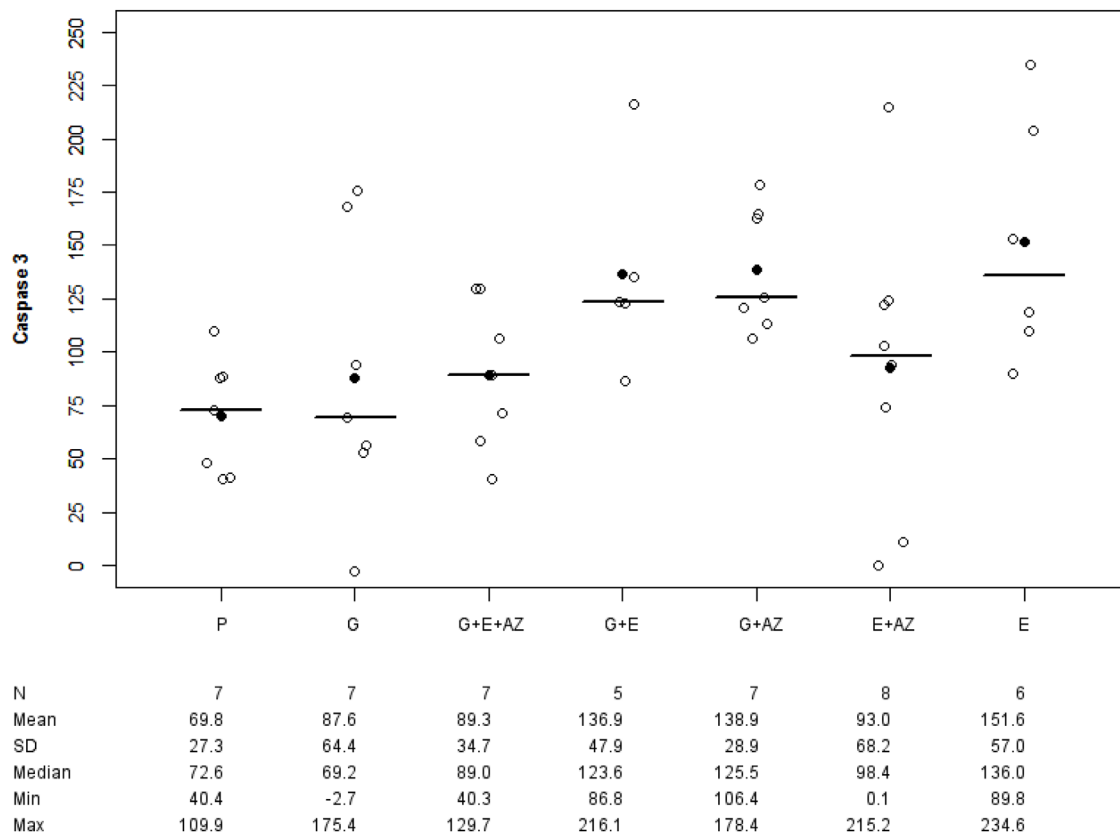


Figure 5: Caspase-3 expression in BxPc3. Placebo (P), Gemcitabine (G), Gemcitabine + Everolimus + Zoledronic acid (G+E+AZ), Gemcitabine + Everolimus (G+E), Everolimus + Zoledronic acid (E+AZ) and Everolimus (E). SD: standard deviation.

Table 3: ERK-p, AKT, hENT1 and Annexin II expression in PANC-1 group

n=26	P n=8	G n=5	G + E + AZ n=7	E + AZ n=6	P
ERK-p					0.040
n	6	4	3	6	
Mean +/-DS	111.8 +/- 65.9	54.0 +/-7.3	69.8 +/- 15.1	142.4 +/- 56.0	
Median	92.4	52.2	74.9	137.1	
Min	45.37	47.57	52.79	80.51	
Max	195.99	63.91	81.67	237.02	
AKT					0.057
n	8	4	3	6	
Mean +/-DS	145.7 +/- 67.8	69.2 +/- 25.6	98.6 +/- 31.6	119.9 +/- 16.5	
Median	136.9	74.8	80.7	127.2	
Min	60.89	33.63	80.05	92.27	
Max	286.53	93.50	135.01	134.36	
hENT1					0.037
n	8	4	4	6	
Mean +/-DS	79.6 +/-22.1	87.7 +/- 13.4	126.6 +/- 33.6	117.7 +/- 29.5	
Median	87.4	91.4	120.5	124.3	
Min	38.39	69.35	95.69	78.58	
Max	102.17	98.91	169.84	159.20	
Annexin II					0.261
n	8	5	4	6	
Mean +/-DS	91.4 +/- 40.9	119.6 +/- 13.8	81.7 +/- 10.4	96.2 +/- 36.2	
Median	90.4	125.5	83.5	105.2	
Min	45.86	97.97	69.38	32.64	
Max	137.89	130.76	90.32	128.78	

Placebo (P), Gemcitabin (G), Gemcitabin + Everolimus + Zoledronic acid (G+E+AZ) and Everolimus + Zoledronic acid (E + AZ)

SD: standard deviation.

the mutated K-ras tumors to chemotherapy. Understanding the pathways involved in PDAC seems insufficient to explain the efficiency of these therapeutics. Moreover, Zhao et al. have shown that the anti-tumor effect of zoledronic acid was mediated partly by the inhibition of angiogenesis [26]. Annexin II seems to be an important factor of gemcitabine resistance through the interruption of the Akt/mTOR pathway [9]. The results of our study coincide with those of Zhao et al., and the expression of Annexin II was significantly under-expressed in the arms treated with everolimus, zoledronic acid and the combination in the BxPc3 group, with an overexpression of caspase-3. The expression of Annexin II is decreased in all groups of PANC-1 as compared to BxPc3, but this expression was not different between PANC-1 groups, so this mechanism seems not to be implied in efficacy of treatments in mt K-ras tumors. Our work confirms the results of a previous study suggesting that Annexin II overexpression is a factor of poor response to gemcitabine [27], especially in wt K-ras tumors. Consequently, a decrease in Annexin II activity would increase response to

gemcitabine, perhaps by modulating the activity of NFkB [28]. Annexin II does not play a role in mt K-ras tumors. Reduction of hENT1 expression is an other resistance factor to gemcitabine [8, 29] and patients with a higher expression of hENT1 seem to have a longer survival. In our study, the impact of hENT1 is not clear because its expression was decreased in gemcitabine plus everolimus and everolimus as compared to placebo or gemcitabine in wt K-RAS tumors without impact of efficacy. In gemcitabine-resistant tumors, hENT1 expression was higher in gemcitabine plus everolimus and zoledronic acid and in everolimus plus zoledronic acid as compared to placebo without significant difference in comparison to gemcitabine maybe by a lack of power. That explains probably not the antitumoral action of these treatments because everolimus and zoledronic acid have not an action on and by hENT1. Their efficacy is probably due to a specific mechanism induced by everolimus and zoledronic acid combination, unknown at this time. This may explain the negativity of II studies evaluating the action of everolimus in K-ras-possibly mutated tumors [17, 30].

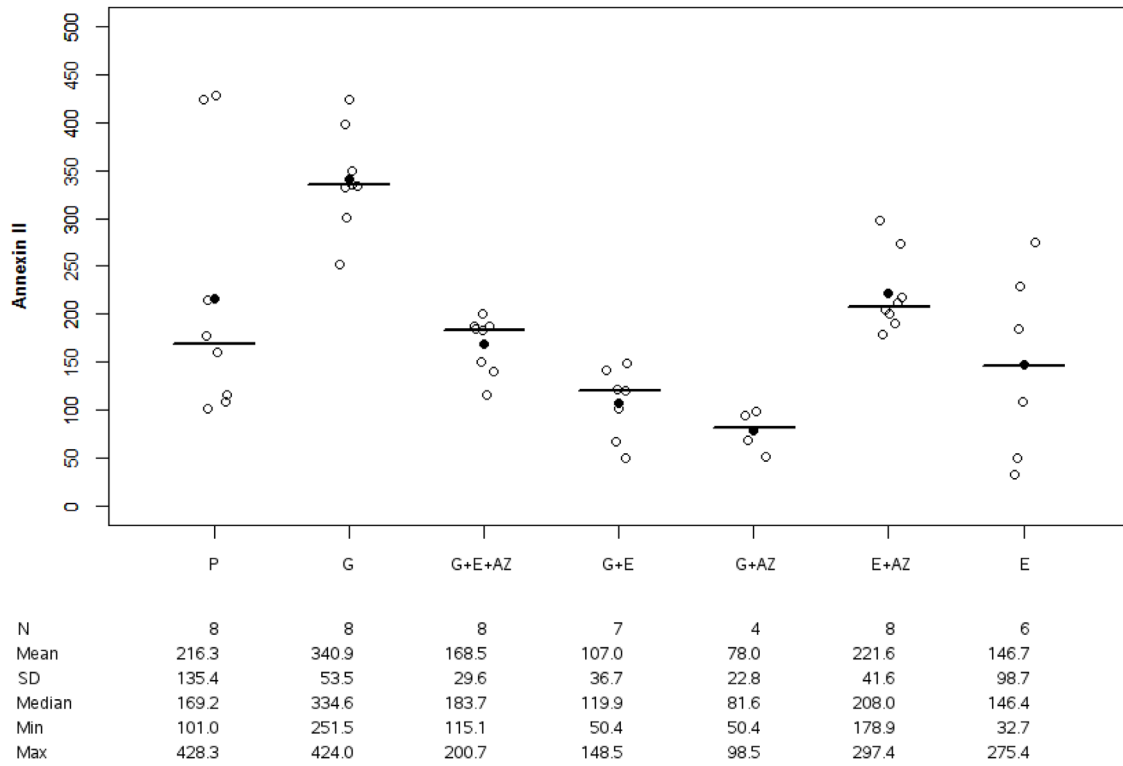


Figure 6: Annexin II expression in BxPc3. Placebo (P), Gemcitabine (G), Gemcitabine + Everolimus + Zoledronic acid (G+E+AZ), Gemcitabine + Everolimus (G+E), Gemcitabine + Zoledronic acid (G + AZ), Everolimus + Zoledronic acid (E+AZ) and Everolimus (E). SD: standard deviation.

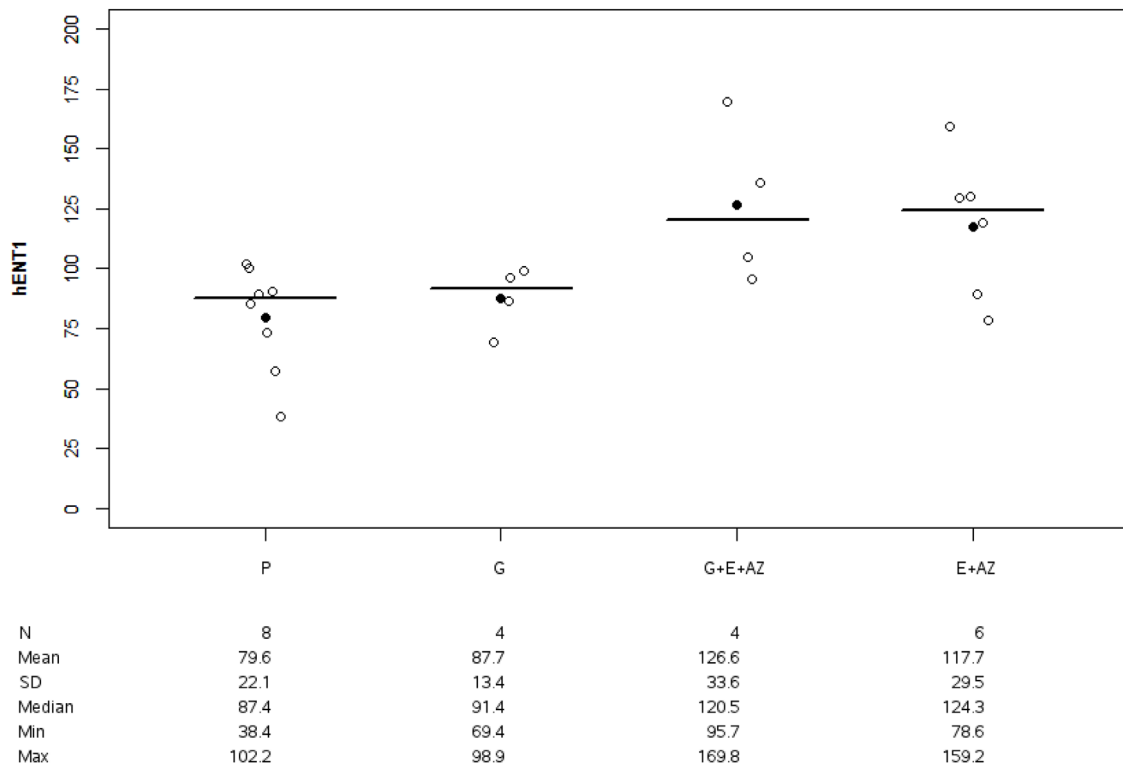


Figure 7: HENT1 expression in PANC1. Placebo (P), Gemcitabine (G), Gemcitabine + Everolimus + Zoledronic acid (G+E+AZ) and Everolimus + Zoledronic acid (E + AZ). SD: standard deviation.

Our study shows that the combination of everolimus and zoledronic acid appears to be effective in adenocarcinoma pancreatic with a best response in mutated K-ras tumors. The mechanisms implied in efficacy of treatments seem to be different according to gemcitabine sensitivity and K-ras mutation. The decreased expression of Annexin II and apoptosis seem to be important in wt K-ras tumors. The mechanisms involved were not fully understood and further clinical trials are necessary. The findings may be relevant for the use of these products in future trials.

MATERIALS AND METHODS

Animals

All experiments involving animals were conducted in accordance with European regulations on the protection of animals used for scientific purposes (Directive 2010/63/EU). The Ethics Committee of Animal Experimentation of Pays de la Loire (No approved the protocol. CEEA.2011.27).

Eight-week old male athymic nude mice (BALB/cAnNRJ –Foxn1nu/Foxn1nu) were obtained from Janvier laboratories (Saint-Berthevin, France). The mice were kept under sterile conditions in a humidity and temperature-controlled room with 12-hour alternations of light and darkness, at the animal facility of Angers University Hospital (SCAHU, *Service commun d'animalerie hospitalo-universitaire*). They had free access to food and water.

Cell lines

Human pancreatic cancer cell lines, PANC-1 and BxPC-3, were purchased from LGC Standards, a partner of the American Type Culture Collection (USA). The PANC-1 cell line was cultured in DMEM and BxPC-3 in RPMI-1640; with 10% fetal bovine serum, 1% glutamine, 1% penicillin-streptomycin mixture, and maintained as adherent cultures at 37°C in a humidified atmosphere containing 5% CO₂. The culture was free of mycoplasma and murine pathogenic viruses (MA Bioproducts). The BxPC-3 cells are characterized by a wild-type (Wt) K-Ras status and sensitivity to gemcitabine, whereas PANC-1 cells present a K-Ras mutation (mt K-ras) and are resistant to gemcitabine.

Tumor implantation and *in vivo* tumor growth

Studies were performed on a nude mouse xenograft model, as previously described [31]. After a period of adaptation, implantations were performed on 6-week old nude mice weighing 20–25g at the time of cell inoculation. Human pancreatic adenocarcinoma cells were suspended in serum-free medium, and 50 µl of cell suspension containing 3 x 10⁶ BxPc3 cells or 4 x 10⁶ Panc-1 cells were subcutaneously injected into the flank of the mice.

The tumor measurements (i.e. length and width) were completed using a vernier caliper, and tumor volume was calculated using the formula for a prolate ellipsoid: {Length (mm) x [width (mm)]² x 0.5, assuming specific gravity to be one and π to be three.

Treatments and drugs

Once the tumor volume had reached 100 mm³, the mice with the two cell lines were randomized to experimental groups for 28 days of treatment.

Consequently, for BxPC-3 cells, the mice were divided into 7 experimental treatment groups: placebo (P), Gemcitabin (G), Everolimus (E), Gemcitabin + Everolimus (G+E), Gemcitabin + Zoledronic acid (G + AZ), Everolimus + Zoledronic acid (E+AZ) and Gemcitabin + Everolimus + Zoledronic acid (G+E+AZ). For Panc-1 cells, the mice were randomized into only 4 experimental treatment groups: placebo (P), Gemcitabin (G), Everolimus + Zoledronic acid (E+AZ) and Gemcitabin + Everolimus + Zoledronic acid (G+E+AZ).

Gemcitabine (GEMZAR, Eli Lilly, France) was administered by peritoneal injection (PI), twice a week at 100 mg/kg of body weight. Zoledronic Acid (Zometa®, Novartis Pharma, France) was administered once a week by PI at 100µg/kg of body weight, and Everolimus (Afinitor®, Novartis Pharma, France) was administered by gavage at 3 mg/kg of body weight. Treatments were provided and prepared by the pharmacy of Angers University Hospital. Control animals in placebo groups received an equal volume of saline solution to the active molecule [32, 33].

Analysis process

Experiments were carried out for 28 days. Upon completion of the experiment, intracardiac blood sampling was conducted in anesthetized mice by isoflurane gas. Subsequently, the animals were euthanized, the tumors were excised and weighted, and the tumor volume was measured.

Histopathological analysis

Following tumor excision, a sample was fixed in 4% neutral buffered formalin, routinely processed and paraffin-embedded. Histopathological analysis was performed using conventional hematoxylin and eosin staining of tissue sections. Evaluation of tumor cell proliferation in primary tumors was performed using Ki67 (DAKO, M7240-MIB France) staining by immunohistochemistry.

Immunoblot analysis

Protein extracts from tumor tissue samples were prepared by suspending the cells in a cell lysis buffer

(Sigma) and protease inhibitor cocktail (Roche). Each extract was prepared as above and an equivalent to 20 µg total protein was separated by SDS-PAGE.

Annexin II Antibody (ab41803, Abcam[®], France); hENT1 (antibody LS-C178673, LSBio[®], France); AKT (ab8932, Abcam[®], France); ERK-p (9101S Cell signaling, France); Raf-1 (ab173539, Abcam[®], France); mTOR (ab1093, Abcam[®], France); Caspase-3 (ab47131, Abcam[®], France). Horseradish peroxidase-conjugated anti-mouse (Promega W402B 28570702[®], France) or anti-rabbit IgG (w401B 29303402[®] Promega, France) was used to detect specific proteins.

Detection of specific proteins was carried out using an enhanced chemiluminescence western blotting kit (Pierce).

We measured the intensity of each band using LAS 4000 software and calculated the relative protein levels normalized to that of the β-actin antibody (A5316-2ML SIGMA[®], France).

Statistical analysis

We used descriptive statistics (mean, standard deviation (SD), median, minimum and maximum values) and doxplots for comparing distributions of quantitative variables across experimental groups. Differences between groups were assessed using the non-parametric Wilcoxon-Mann-Whitney tests and when the results were significant ($p < 0.05$), the comparison 2 to 2 were assessed using Kruskal-Wallis test to compare combined treatments. Significance was set at $p \leq 0.05$.

Statement of translational relevance

Everolimus used as a single agent has not an anti-tumor effect in patients with gemcitabine-refractory metastatic pancreatic adenocarcinoma. This study has shown a new therapeutic approach for treatment of pancreatic adenocarcinoma and the synergic efficacy of zoledronic acid, everolimus and gemcitabine. We would realize a phase I-II study to evaluate the efficacy of combination of these three treatments in pancreatic adenocarcinoma with K-ras mutation and Annexin II overexpression. The aim of this study would be increase objective response to gemcitabine from 5 to 15%. We would test everolimus 5 and 10 mg in first treatment.

CONFLICTS OF INTEREST

Novartis.

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