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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)

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Abstract

The majority of cancers are caused by mutations of a few signal transducers such as the GTPase RAS, the kinase Src and the tumor suppressor p53. Thus, a group of specific chemical compounds called 'signal therapeutics', that block or reverse selectively these abnormally activated signaling pathways would be very useful for the treatment of these signally disordered cancers. More than 90% of human pancreatic cancers are associated with oncogenic mutations of RAS, in particular K-RAS at codon 12. We have previously shown that, PAK1, the Rac/CDC42-dependent Ser/Thr kinase, is essential for RAS/estrogen-induced transformation and neurofibromatosis (NF). Furthermore, we and others have demonstrated that the growth of mouse RAS-induced sarcomas allografts in mice is almost completely suppressed by either FK228 or a combination of two complimentary Tyr-kinase inhibitors, PP1 and AG 879, all of which block the RAS-induced activation of PAK1. Since, so far no effective therapeutic is available for the treatment of pancreatic cancer patients, we have examined the therapeutic potential of either FK228, the combination of these two Tyr-kinase inhibitors or GL-2003, a water-soluble derivative of AG 879, on human pancreatic cancer (Capan-1) xenograft in mice. Among these PAK1-blocking approaches, the PP1/GL-2003 combination is the most effective in the therapy of this cancer xenograft model. Its therapeutic potential is equivalent to those of gemcitabine and kigamicin D which suppress by 70–80% the growth of a similar human pancreatic cancer xenograft model. Also, this PP1/GL-2003 combination therapy has been proven to be very effective to suppress the estrogen-independent growth of an *NF1*-deficient multidrug/FK228-resistant human breast cancer (MDA-MB-231) xenograft in mice.

Keywords: Pancreatic cancers; RAS; PAK1; PP1; GL-2003; Neurofibromatosis

1. Introduction

Every year around 600,000 people on this planet die of pancreatic cancers. The collective median survival time of all pancreatic cancer patients is 4–6 months. The overall 5-year survival rate for this disease is less than 5%. The main reason for such a poor survival rate is that so far no clinically effective therapeutic has been developed for this disease. Around 30% of all human cancers carry oncogenic RAS mutations, and most notably more than 90% of human pancreatic cancers are associated with such mutations, in particular K-RAS at codon 12 [1], which causes the constitutive

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activation of the G protein RAS, eventually leading to the abnormal activation of PAK1, a Rac/CDC42-dependent Ser/Thr kinase, or another Ser/Thr kinase called AKT/PKB, through the PI-3 kinase cascade [2,3]. An anti-biotic called kigamicin D, which blocks the PI-3 kinase/AKT pathway, strongly suppresses the growth of human pancreatic cancer xenografts in mice, suggesting that this pathway might be one of the selective targets for anti-pancreatic cancer drugs [3].

FK228, the most potent histone deacetylase (HDAC) inhibitor so far discovered, has been shown to suppress the growth of both v-Ha-RAS-induced sarcoma allograft [4], and human pancreatic cancer cell lines such as Capan-1 in cell culture (IC50: 3 nM) which carry both K-RAS and p53 mutants [5]. Furthermore, we and others found that (i) PAK1 is essential for RAS-transformation [2,6], that (ii) the growth of RAS-induced sarcoma allograft in mice is almost completely suppressed by a combination of two complementary Tyr-kinase inhibitors, PP1 and AG 879, which block PAK1 activation by inhibiting a Src family kinase(s) and ETK, respectively [2,7,8], with their IC50 around 10 nM, and that (iii) FK228 blocks the activation of PAK1 in RAS transformants and estrogen-dependent breast cancer cells [9]. Based on these previous observations, we have examined the therapeutic potential of FK228 alone, a combination of PP1 with either AG 879 or its water-soluble derivative called GL-2003 on the growth of Capan-1 xenograft in mice.

2. Materials and methods

2.1. Materials

FK228 was supplied from Astellas Pharma (former Fujisawa Pharmaceuticals) as previously described [10]. PP1 and AG 879 were synthesized as previously [2]. GL-2003, a water-soluble derivative of AG 879, was synthesized via GL-2002 [8], and the detail of their synthesis will be described in Appendix A1 (supplement information). In brief, as shown in Fig. 1, GL-2002 (compound **4**) was deprotected with $SnCl_4$ in AcOEt, yielding GL-2003 (compound **5**).

The human pancreatic cancer cell line Capan-1 and human breast cancer cell line MDA-MB-231 were obtained from ATCC, and grown in RPMI-1640 containing 15 and 10% fetal calf serum (FCS), respectively, under standard culture conditions. BALB/c nu/nu mice (female, 7-week-old) were purchased from Animal Resources Center (Western Australia).

2.2. Effect of FK228 on the growth of MDA-MB-231 cell line in vitro

MDA-MD-231 (2×10^3 cells) cells of MDA-MB-231 were seeded in a 96-well plate and incubated in the culture medium RPMI-1640 containing 10% fetal calf serum with or without FK228 (10 pM–1 nM) for a week, and cells were counted by a hemo cytometer. FK228 at 100 and 300 pM inhibited their growth by 62 and 100%, respectively, while at 10 pM had little effect on their growth.

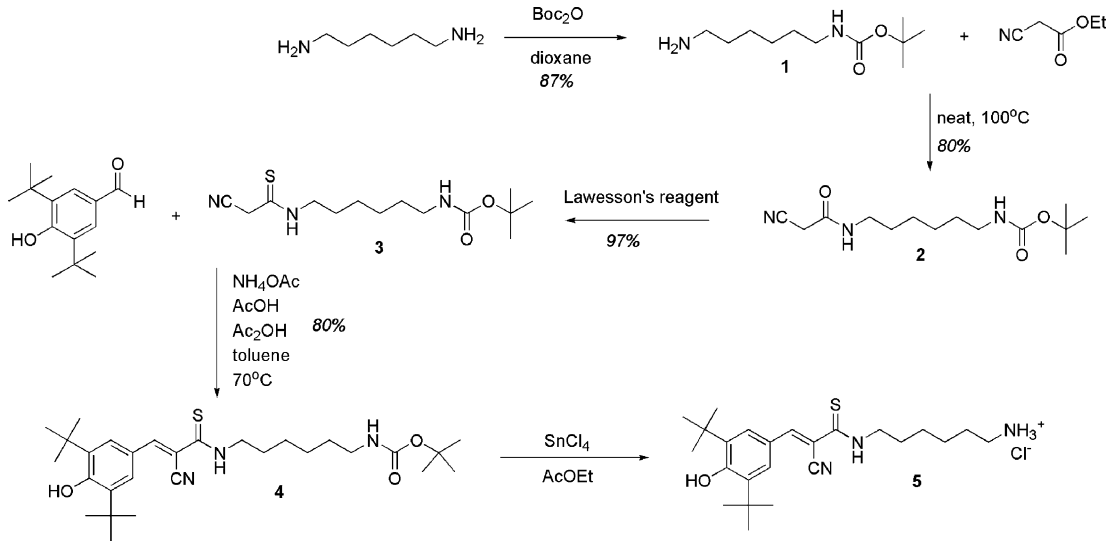

Fig. 1. GL-2003 (compound **5**): its chemical structure and synthesis.

2.3. In vitro bioassay of GL-2003 using human RAS-transformants

The human colon cancer cell line LIM-1899 carries an oncogenic mutant of K-RAS in which Gly12 is replaced by Ala [11]. The effect of GL-2003 was compared on the kinase activity of PAK1 in both the LIM-1899 and *NF1*-deficient (MDA-MB-231) cells with that of AG 879 at the indicated concentrations, using the methods previously described [8,9]. The protein levels of PAK1 were monitored by immuno-blotting with the anti-PAK1 anti body. Using LIM-1899, it was further examined whether the combination of GL-2003 (100 nM) and PP1 (10 nM) show any additive or synergetic effect to inhibit PAK1 activation. In addition, the effect of AG 879/GL-2003 (10–100 nM) and PP1 (10 nM) on the anchorage-independent growth of LIM-1899/Capan-1 was also examined in soft agar using the procedures as described previously [8].

2.4. Xenograft experiments

Capan-1 (5×10^6 cells per mouse) in 0.1 ml of culture medium were injected sub-cutaneously into several groups of 8–9 nude mice. Three or eight days later, when the average tumor size reached around 5 mm in diameter, each group was treated intra-peritoneally with either FK228 (1.5 mg/kg), GL-2003 (20 mg/kg), a combination of PP1 and AG 879 (20 mg/kg of each drug), a combination of PP1 and GL-2003 (20 mg/kg of each drug), or vehicle alone (0.1 ml of 1% DMSO in PBS) as the control twice a week for 2–3 weeks.

MDA-MB-231 (5×10^6 cells per mouse) in 0.1 ml of a culture medium containing 50% Matrigel (BD Biosciences, Bedford, MA) were injected sub-cutaneously into three groups of 8–9 nude mice. Eighteen days later, when the average tumor size reached around 5 mm in diameter, each group was treated intra-peritoneally with either FK228 (1.5 mg/kg), a combination of PP1 and GL-2003 (20 mg/kg of each drug), or vehicle alone (0.1 ml of 1% DMSO in PBS) as the control twice a week for 2–3 weeks. Palpable tumor diameters were measured twice a week. Tumor volumes were calculated as: $(\pi/48)\times[\text{length (mm)}+\text{width (mm)}]^3$.

Under these conditions, none of mice suffered from any detectable adverse effect from any of these test drugs. It should also be noted that, according to the Australia's strict regulation of animal experimentation, all the mice carrying solid tumors larger than 1 cm in diameter were immediately terminated for the animal welfare.

2.5. Statistical analysis

Data are expressed as means ± SD (standard deviation), unless otherwise stated. Statistic analysis was performed mainly by means of Student's *t*-test. Differences were considered significant at $P<0.05$.

3. Results and discussion

3.1. PP1 and GL-2003 cause a synergetic effect in the therapy of human pancreatic cancer xenograft in mice

For the combination therapy of PP1 and AG 879, we followed the exact dose (20 mg/kg of each, i.p. twice a week) as we used previously for testing on RAS-induced sarcoma allograft in mice [2]. For FK228 therapy, we chose a mean dose (1.5 mg/kg, i.p. twice a week), between 1 mg/kg for breast cancers xenografts and 2.5 mg/kg for NF xenograft in mice [10,12]. As shown in Fig. 2, both FK228 alone or the PP1/AG 879 combination showed the almost same anti-cancer potential, that is around 50% inhibition of their growth.

One of the problems associated with this combination therapy is that neither PP1 nor AG 879 are water-soluble, and therefore, we have to administer each drug in suspension. However, since we have found that chemical modification of AG 879 at the amino group does not affect its anti-PAK1 activity in cells [8], we have developed a water-soluble derivative of AG 879, called GL-2003, simply by *N*-hexylamination (for its chemical structure and synthesis, see Fig. 1). As shown in Fig. 3, the anti-PAK1 activity of GL-2003 is basically indistinguishable from that of AG 879 in human cancer cells.

To test the anti-cancer potential of GL-2003 (20 mg/kg, i.p. twice a week), we examined the therapeutic effect of GL-2003 alone and its

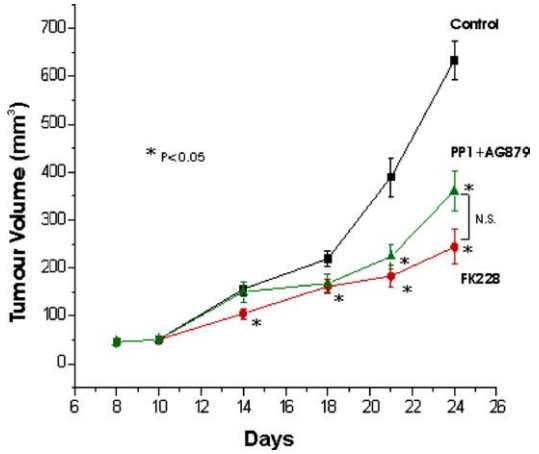

Fig. 2. Effect of FK228 alone and 'PP1 plus AG 879' on pancreatic cancer xenograft. Eight days after the inoculation of Capan-1 cells, mice were treated with either 'PP1 plus AG 879', FK228 or the vehicle alone as described under Section 2.

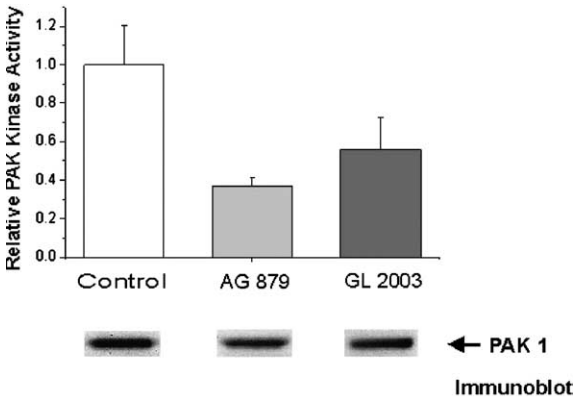

Fig. 3. Anti-PAK1 effects of GL-2003 compared with AG 879. MDA-MB-231 cells were treated with GL-2003 or AG 879 at 10 nM and their PAK1 kinase activity and PAK1 protein levels were measured as described under Section 2.

combination with PP1 on the same pancreatic cancer xenografts in mice. The GL-2003/PP1 combination suppressed their growth by around 80% (see Fig. 4A), while FK228 alone showed only 50% inhibition (see Fig. 4A). Surprisingly, PP1 or GL-2003 alone has no statistically significant effect (see Fig. 4B and C), while the combination of FK228 with PP1 appears to be less effective than FK228 alone (Fig. 4C). These results suggest that (i) the combination of GL-2003 and PP1 causes a remarkable 'synergy' in the therapeutic effect, and that (ii) the water-soluble GL-2003 is clearly more effective than either the water-insoluble AG 879 (see Fig. 2) or FK228 (see Fig. 4C) in potentiating the anti-pancreatic cancer activity of PP1.

Interestingly, unlike this 'human' pancreatic cancer xenograft, in the case of v-Ha RAS-transformed 'mouse' sarcoma (NIH/3T3 fibroblast) allograft, PP1 or AG 879 alone suppresses their growth by around 50%, while the combination of PP1 and AG 879 causes an almost complete inhibition of their growth [2,7]. These results suggest that although the RAS/PAK1 pathway is activated in both cases, a subtle difference in their genetic background between these two RAS transformants appears to affect their susceptibility to each of these Tyr-kinase inhibitors.

The therapeutic potential of this PP1/GL-2003 combination appears to be equivalent to those of two potent drugs, gemcitabine (80 mg/kg, i.p. twice a week) and kigamicin D (0.8 mg/kg, oral administration, daily), which cause 70–80% inhibition of the growth of human pancreatic cancer xenografts in mice [3,13].

3.2. PP1/GL-2003 combination is also effective on the growth of estrogen-independent growth of NF1-deficient multidrug-resistant human breast cancer xenograft

Previously, we have shown that the anti-PAK drug FK228 markedly inhibits the estrogen/PAK1-dependent growth of human breast cancer xenograft in mice,

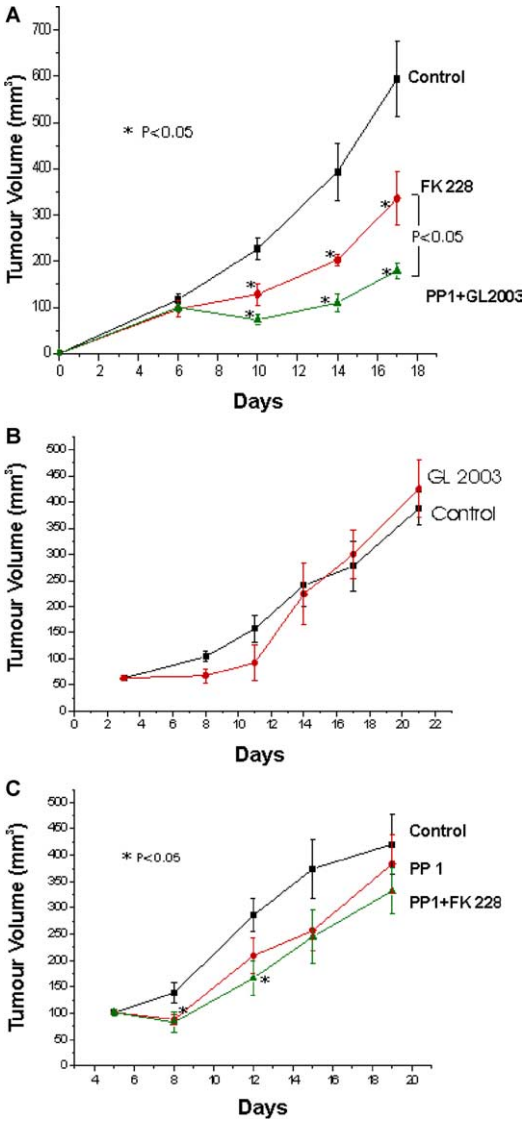

Fig. 4. A. Effect of 'PP1 plus GL-2003' on pancreatic cancer xenograft. Three days after the inoculation of Capan-1 cells, mice were treated with either 'PP1 plus GL-2003', FK228 or the vehicle alone as described in Fig. 2. (B) GL-2003 alone has no effect on pancreatic cancer xenograft. The Capan-1 bearing mice were treated with GL-2003 or the vehicle alone as described in (A). (C) Effect of PP1 alone or its combination with FK228. The Capan-1 bearing mice were treated with PP1 (plus or minus FK228) or the vehicle alone as described in (A).

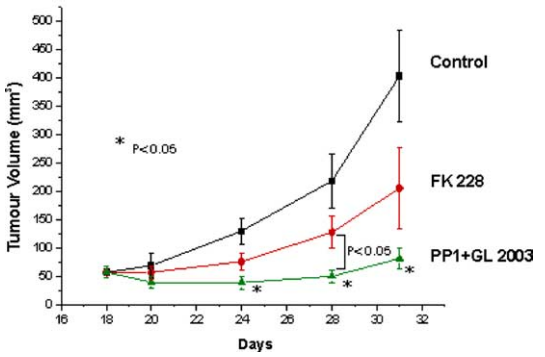

Fig. 5. Effect of 'PP1 plus GL-2003' on *NF1*-deficient cancer xenograft. MDA-MB-231 bearing mice were treated with either 'PP1 plus GL-2003', FK228 or the vehicle alone as described under Section 2.

using a tamoxifen-resistant sub-clone of MCF-7 [9,12]. To further determine whether the estrogen-independent growth of breast cancers is also susceptible to FK228, we have used an *NF1*-deficient multidrug-resistant human breast cancer called MDA-MB-231. The IC50 of FK228 for this cell line is around 100 pM (for detail, see Section 2), which is 20 times higher than the IC50 for MCF-7 (around 5 pM) [9], suggesting that this cancer cell line is FK228-resistant. In xenograft in mice, FK228 (1.5 mg/kg, i.p. twice a week) suppressed the growth of this cell line only by around 50%, while the combination of PP1 and GL-2003 (20 mg/kg of each drug, i.p. twice a week) almost completely (by more than 90%) blocked its growth (see Fig. 5). The result suggests that this new combination therapy would be a promising alternative of FK228 to treat both estrogen-independent multidrug-resistant breast cancers and *NF1*-deficient tumors (neurofibromatosis type 1).

3.3. No in vitro synergy between GL-2003 and PP1

Although, the 'PP1 and GL-2003' synergy was clearly observed in vivo, so far there is no in vitro evidence supporting such a synergy in blocking the RAS-induced PAK1 activation (see Fig. 6). Furthermore, although PP1 (10 nM) or AG 879 (10–100 nM) alone clearly blocks the anchorage-independent growth of RAS-transformants in soft agar, the effect of their combination exceeds only marginally that of each drug alone (see Table 1). These results suggest that this synergy might be achieved only under unique in vivo conditions.

Regarding the possible molecular mechanism underlying this synergy between GL-2003 and PP1, we speculate that the apparent synergy might be due to an emerged drug-resistance of the RAS transformants. It could be induced by each drug that would up-regulate distinct multi-drug-resistant (MDR) proteins, pumping each drug out of the cells. Alternatively, the Capan-1 cell line could contain a tiny population of two distinct drug-resistant mutants/variants: one is resistant to PP1, and the other GL-2003-resistant. Thus, in an early stage of the tumor development in mice, either PP1 or GL-2003 alone actually showed its therapeutic effect, if not so strong (see Fig. 4B and C). However, eventually at the later stage, it appears that all these sensitive

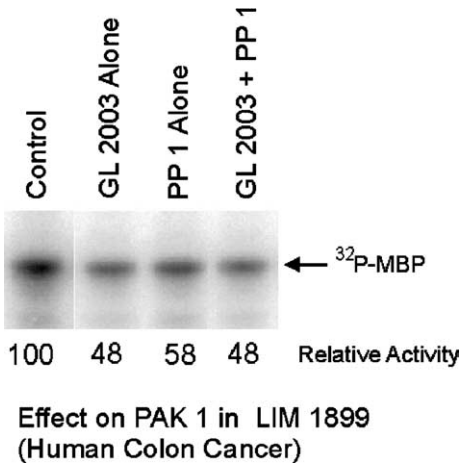

Fig. 6. No synergy between GL-2003 and PP1 in vitro. LIM-1899 cells were treated with either GL-2003 (100 nM) or PP1 (10 nM) alone or their combination, and the PAK1 kinase activity was measured as described in Fig. 3.

Table 1
No synergy between AG 879 and PP1 in inhibiting the colony formation in soft agar

Drug added	Colonies per plate (%)	Inhibition (%)
Control (no drug)	372 (100)	0
AG 897 (10 nM)	234 (62)	38
PP1 (10 nM)	215 (57)	43
AG 879 (10 nM)+ PP1	186 (50)	50
AG 879 (100 nM)	215 (57)	43
AG 879 (100 nM)+ PP1	186 (50)	50

2×10^4 cells of LIM-1899 were seeded in each well of soft agar, incubated with indicated concentrations of AG 879 or PP1, and the number of colonies (containing more than 20 cells) formed after 2 weeks was counted as described under Section 2. The presented data were the average of those from three wells for each point, and the standard deviation was less than 5%.

population perished, and only the remaining PP1-resistant or GL-2003-resistant population took over the whole tumor. In the combination of two drugs, the growth of PP1-resistant population would be blocked by GL-2003, while that of GL-2003-resistant population would be suppressed by PP1. In this context, it is worth noting that the combination of FK228 and PP1 is clearly less effective than FK228 alone in vivo (Fig. 4C). Recently, FK228 was found to up-regulate MDR1 which pumps out a variety of drugs including FK228 [14]. Perhaps, PP1 might also up-regulate MDR1 (or another drug-resistance protein), leading to the development of a common drug-resistance in cancers to both FK228 and PP1, but not to AG 879 derivatives such as GL-2003.

We are currently testing the effect of this new combination therapy on several other types of human cancers such as colon and prostate cancers as well as gliomas and *NF2*-deficient tumors (neurofibromatosis type 2), all of which FK228 has previously shown a potent therapeutic effect on, and therefore, are suggested to require PAK1 for their malignant growth [10,12,15,16]. Furthermore, we are under way to develop a water-soluble derivative of PP1 called GL-2005 which would be more suitable for clinical application.

At least two independent reports were previously published, regarding the involvement of Src family kinases in the growth of human pancreatic cancer xenograft in mice. PP2, a PP1-related Src family kinase inhibitor (IC50: 10–20 μM), alone (2 mg/kg, thrice a week) suppresses the growth of gemcitabine-resistant human pancreatic cancer line derived from Panc-1 (mutated in both K-RAS and p53) by only 25% or less, while its combination with gemcitabine (100 mg/kg, thrice a week) suppresses almost completely [17]. AZM475271, another c-Src inhibitor (IC50 around 5 μM), alone (50 mg/kg, daily) can suppress the growth of a metastatic human pancreatic cancer line L3.6p1 derived from COLO 357 (with unknown genetic background) by 40%, while its combination with gemcitabine (100 mg/kg, twice a week) suppresses by 90% [18]. Both these results and our own observation here suggest that these Src inhibitors alone are not sufficient to block completely the growth of pancreatic cancers in general.

Actually, the primary target of PP1 which is required for the RAS-induced activation of PAK1 is not c-Src, but an un-identified member of Src family kinases, because the IC50 of PP1 is around 10 nM to inhibit both PAK1 activation in RAS transformants and their anchorage-independent growth (and not their anchorage-dependent growth) [7]. Furthermore, PP1 is not cytotoxic at all even at 10 μM, on either RAS-transformed or normal fibroblasts [7]. The same is true for AG 879/GL-2003 (IC50: 5–10 nM). The primary target of AG 879/GL-2003 is a Tyr-phosphorylated protein of 62 kDa which is required for the ETK–PAK1 interaction and PAK1 activation [8]. Even at 100 nM, AG 879/GL-2003 has no effect on the anchorage-dependent growth of RAS transformants, while inhibiting their anchorage-independent growth at even 5 nM [8]. In addition, PAK1 is known to be essential for both angiogenesis and VEGF expression [19,20]. Thus, it is most likely that the combination of PP1 and GL-2003 suppresses the growth of RAS-induced pancreatic and NF1-deficient breast solid cancer xenografts by blocking both the PAK1-dependent anchorage-independent cell growth per se and angiogenesis. To confirm this notion, we are currently performing the pharmacokinetics of both PP1 and GL-2003 to monitor their concentrations in both circulation and target tumors, during the first 3 days.

Ideally speaking, the 'direct' PAK1 inhibitors would be much more straightforward approaches to the signal therapy of these PAK1-dependent NF and cancers which represent more than 70% of all human cancers [9]. However, so far only one PAK1-specific inhibitor called CEP-1347 is available, and its IC50 (around 1 μM) would be considered too high to test its therapeutic effect on either animal models or cancer patients [21]. In fact its clinical trials (phases 2/3) for Parkinson's disease were recently discontinued. So we are currently developing a much more potent PAK1-inhibitor called ST-2004, a very specific derivatives of a marine compound called ST-2001, which is distantly related to CEP-1347, but whose IC50 for PAK1 is around 1 nM [21].

Acknowledgements

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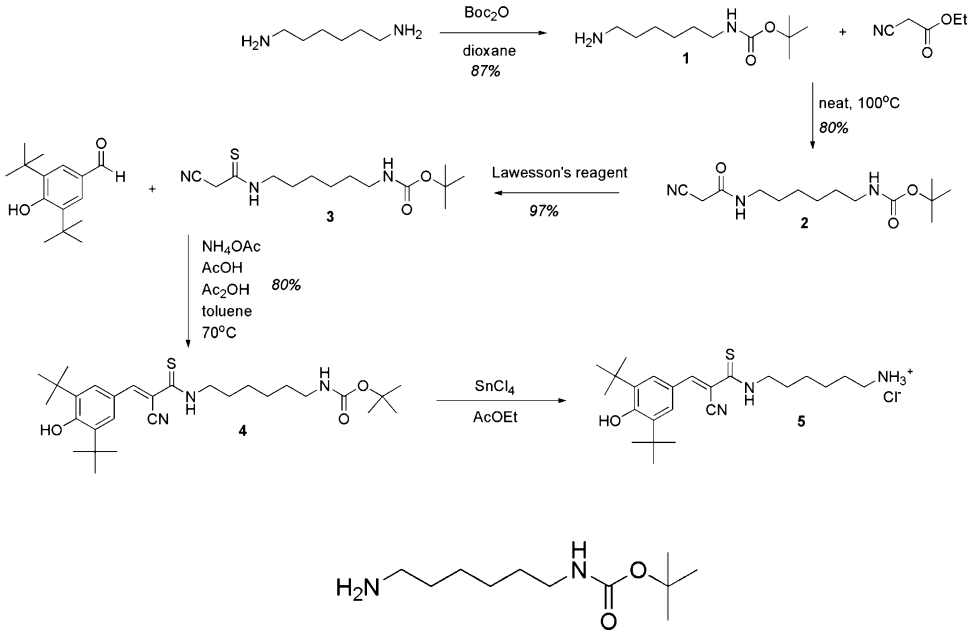
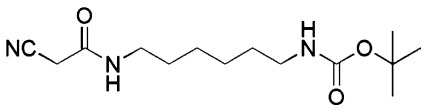
Appendix/supplement. Procedures for the synthesis of AG-879 hexylamine derivative (GL-2003)

Anhydrous dioxane was purchased from Aldrich, THF was dried over sodium/benzophenone, toluene

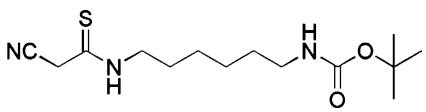
was distilled over P_2O_5 and stored over sodium wires, ethyl acetate used in reaction was dried over activated 4 Å sieves. Reagents were used as purchased without further purification. Purification was performed by flash chromatography using AR grade solvent. NMR spectra were recorded on a Bruker 200 MHz instrument. LC/MS analysis was performed on a Finnigan LCQ Advantage Max instrument.



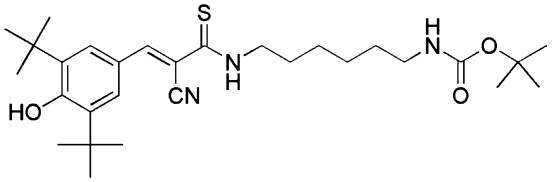
1 [22]: A solution of Boc_2O (6.06 g, 27.7 mmol) in 75 ml of dioxane was added dropwise at room temperature to a solution of 1,6-diaminohexane (25 g, 2151 mmol) over a 2.5 h period. The reaction was then stirred at room temperature for 22 h. After this time, the reaction mixture was concentrated in vacuo and the residue washed with water. The aqueous layer was filtered in order to remove the insoluble white material. The aqueous layer was then extracted three times with dichloromethane. The combined organic layers were washed two times with water, dried over Na_2SO_4 and concentrated to afford 1 as a light yellow thick oil (5.23 g, 87%). NMR ^1H ($CDCl_3$, ppm): 1.26 (m, 2H); 1.30–1.35 (m, 4H); 1.44 (s, 9H); 1.49–1.44 (m, 2H); 2.68 (t, 2H, J = 4.4 Hz); 3.10 (q, 2H, J = 4.3 Hz); 4.5 (br s, 1H).



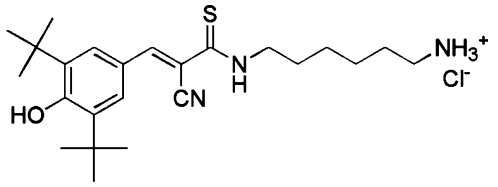
2 A mixture of 1 (5.23 g, 24.2 mmol) and ethylcyanoacetate (2.57 ml, 24.2 mmol) was heated to 100 °C with stirring. After 2 h, the reaction was cooled down to room temperature during which time the resulting dark oil solidified. This solid was purified by flash chromatography (SiO_2, CH_2Cl_2/AcOEt 60:40). A white solid was obtained (m = 5.5 g, 80%). NMR ^1H ($CDCl_3$, ppm): 1.34–1.37 (m, 4H); 1.45 (s, 9H); 1.45–1.58 (m, 4H); 3.12 (t, 2H, J = 4.4 Hz); 3.30 (q, 2H, J = 4.6 Hz); 3.37 (s, 2H); 4.5 (br s, 1H); 6.35 (br s, 1H).



3 [23]: Lawesson's reagent (3.85 g, 9.53 mmol) was added to a solution of 2 (5.4 g, 19.05 mmol) in dry THF at room temperature. The reaction was stirred for 2 days at room temperature. After this time, TLC (SiO_2, AcOEt/Pet. Et. 60:40) indicated complete conversion. The reaction was then concentrated in vacuo and the residue dissolved in CH_2Cl_2 and concentrated onto silica gel. Purification was then performed by flash chromatography (SiO_2, AcOEt/Hex. 20:80–40:60). A yellowish solid was obtained (m = 5.53 g, 97%). NMR ^1H ($CDCl_3$, ppm): 1.40 (m, 4H), 1.45 (s, 9H); 1.45–1.52 (m, 2H); 1.71 (qt, 2H, J = 4.4 Hz); 3.14 (t, 2H, J = 4.4 Hz); 3.68 (q, 2H, J = 4.2 Hz); 3.92 (s, 2H); 4.6 (br s, 1H); 8.22 (br s, 1H).



4 [24]: A mixture of 3,5-terbutyl-4-hydroxybenzaldehyde (2.99 g, 12.2 mmol), 3 (5.5 g, 18.4 mmol), NH_4OAc (8 g, 104 mmol), AcOH (3.5 ml, 61.2 mmol), Ac_2O (6.92 ml, 73.4 mmol) in 20 ml of toluene was heated at 70 °C for 2 h. After this time, TLC (SiO_2, AcOEt/Pet. Et. 20:80) indicated completion of the reaction. The reaction mixture was then dissolved in CH_2Cl_2 and poured onto water. The aqueous layer was extracted three times with CH_2Cl_2.The combined organic layers were washed with water and brine, dried over Na_2SO_4 and concentrated. The residue was redissolved in CH_2Cl_2 and concentrated onto silica gel. Purification was performed by flash chromatography (SiO_2, AcOEt/Pet. Et. 10:90–20:80). A foamy and glassy orange solid was obtained (m = 5.05 g, 80%). NMR ^1H ($CDCl_3$, ppm): 1.45 (s, 9H); 1.48 (s, 18H); 1.45–1.51 (m, 6H); 1.75 (qt, 2H, J = 4.8 Hz); 3.12 (t, 2H, J = 4.4 Hz); 3.85 (q, 2H, J = 3.8 Hz); 4.5 (br s, 1H); 5.7 (br s, 1H), 7.90 (s, 1H); 8.77 (s, 1H).



5: $SnCl_4$ (0.447 ml, 3.88 mmol) was added to a solution of 4 (1 g, 1.94 mmol) in 20 ml dry AcOEt. The reaction was stirred overnight. After this time, the reaction mixture was concentrated in vacuo. The residue was purified by flash chromatography (SiO_2, CH_2Cl_2/MeOH 90:10). The yellow solid obtained was further triturated in a mixture Et_2O/Hexane, filtered and dried in vacuo. NMR ^1H (DMSO d_6, ppm): 1.34 (m, 4H); 1.40 (s, 18H); 1.54 (m, 2H); 1.65 (m, 2H); 2.76 (q, 2H, 4.4 Hz); 3.61 (q, 2H, J = 4.2 Hz); 7.74 (br s, 3H); 7.81 (s, 1H), 7.91 (s, 1H); 7.93 (s, 1H); 10.28 (m, 1H). MS (ESI+): 416 ($M–Cl^-$).

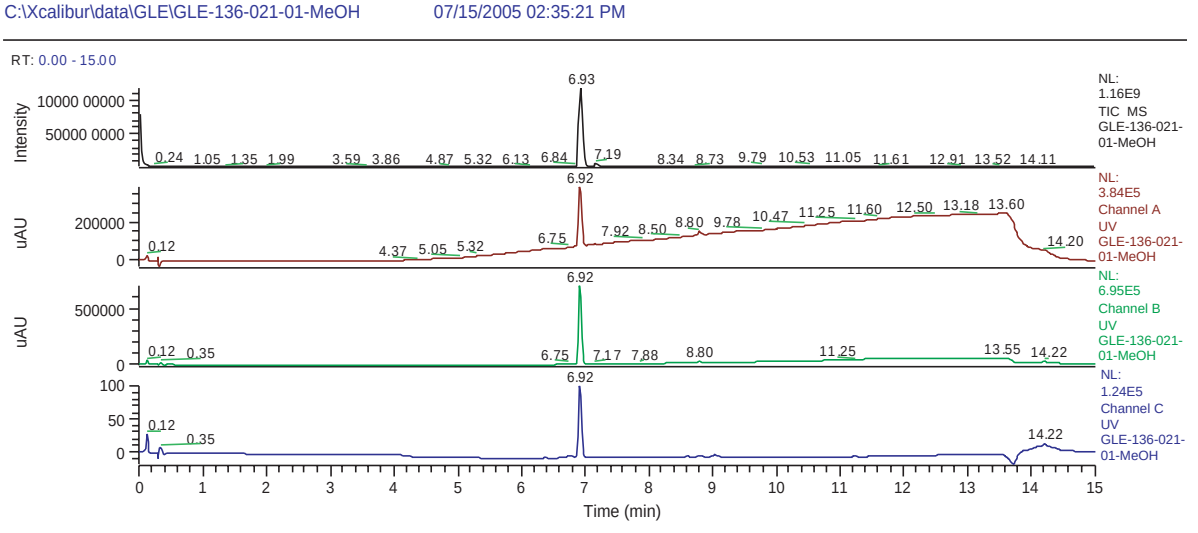
HPLC-MS analysis of final compound GL-2003:

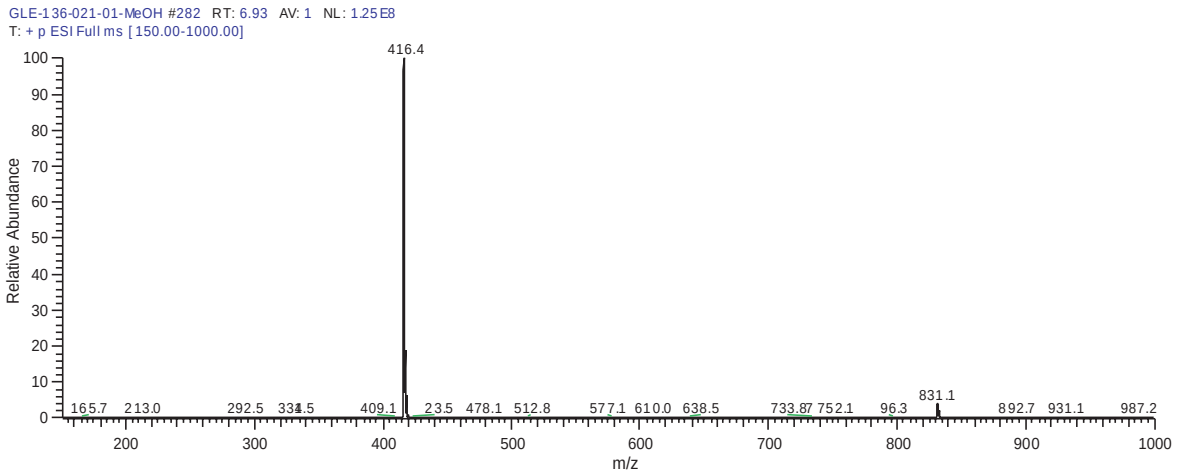
Solvents: A, H_2O + Formic acid 0.1%; B, CH_3CN + Formic acid 0.1%.

Pressure: Minimum (psi), 0.00; Maximum (psi), 6258(Table A1).

Table A1
Gradient:

Time (min)	Flow (ml/min)	*A* (%)	*B* (%)
0.00	1.000	100.0	0.0
2.00	1.000	100.0	0.0
10.00	1.000	0.0	100.0
12.00	1.000	0.0	100.0
12.10	1.000	100.0	0.0
14.00	1.000	100.0	0.0
14.10	0.000	100.0	0.0





References

[1] J. Bos, Ras oncogenes in human cancer: a review, Cancer Res. 49 (1989) 4682–4689.

[2] H. He, Y. Hirokawa, E. Manser, L. Lim, A. Levitzki, H. Maruta, et al., Signal therapy for RAS-induced cancers in combination of AG 879 and PP1, specific inhibitors for ErbB2 and Src family kinases, that block PAK activation, Cancer J. 7 (2001) 191–202.

[3] J. Lu, S. Kunimoto, Y. Yamazaki, M. Kaminishi, H. Esumi, Kigamicin D, a novel anti-cancer agent based on a new anti-austerity strategy targeting cancer cells' tolerance to nutrient starvation, Cancer Sci. 95 (2004) 547–552.

[4] H. Ueda, H. Nakajima, Y. Hori, T. Goto, M. Okuhara, Action of FK228, a novel anti-tumor bicyclic depsipeptide produced by *C. violaceum* #968, on Ha-RAS transformed NIH3T3 cells, Biosci. Biotechnol. Biochem. 58 (1994) 1579–1583.

[5] N. Sato, T. Ohta, H. Kitagawa, M. Kayahara, I. Ninomiya, S. Fushida, et al., FK228, a novel HDAC inhibitor, induces cell cycle arrest and subsequent apoptosis in refractory human pancreatic cancer cells, Int. J. Oncol. 24 (2004) 679–685.

[6] Y. Tang, Z. Chen, D. Ambrose, J. Liu, J.B. Gibbs, J. Chernoff, et al., Kinase-deficient PAK1 mutant inhibits RAS transformation of Rat-1 fibroblasts, Mol. Cell. Biol. 17 (1997) 4454–4464.

[7] H. He, Y. Hirokawa, A. Levitzki, H. Maruta, An anti-RAS cancer potential of PP1, an inhibitor specific for Src family kinases; in vitro and in vivo studies, Cancer J. 6 (2000) 243–248.

[8] H. He, Y. Hirokawa, A. Gazit, Y. Yamashita, H. Mano, Y. Kawakami, et al., The Tyr-kinase inhibitor AG 879, that blocks the ETK–PAK1 interaction, suppresses RAS-induced PAK1 activation and malignant transformation, Cancer Biol. Ther. 3 (2004) 96–101.

[9] Y. Hirokawa, M. Arnold, H. Nakajima, J. Zalcberg, H. Maruta, Signal therapy of breast cancers by the HDAC inhibitor FK228 that blocks the activation of PAK1 and abrogates the tamoxifen-resistance, Cancer Biol. Ther. 4 (2005) 956–960.

[10] Y. Hirokawa, H. Nakajima, O. Hanemann, A. Kurtz, S. Frahm, V. Mautner, et al., Signal therapy of NF1-deficient tumor xenoograft in mice by the anti-PAK1 drug FK228, Cancer Biol. Ther. 4 (2005) 379–381.

[11] H. Davies, G. Bignell, C. Cox, P. Stephens, S. Edkins, S. Clegg, et al., Mutations of the BRAF gene in human cancer, Nature 766 (2002) 1–6.

[12] Y. Hirokawa, M. Arnold, H. Nakajima, H. Maruta, Signal therapy of cancers: blocking PAK pathways by FK228, Proceedings of the Miami Winter Symposium, vol. 16, 2005, p. 64.

[13] C. Yau, J. Wheeler, K. Sutton, D. Hedley, Inhibition of integrin-linked kinase by a selective small molecule inhibitor, QLT0254, inhibits the PI-3 kinase/PKB/mTOR, Stat3, and FKHR pathways and tumor growth, and enhances gemcitabine-induced apoptosis in human orthotopic primary pancreatic cancer xenografts, Cancer Res. 65 (2005) 1497–1503.

[14] J. Xiao, Y. Hunag, Z. Dai, W. Sadee, J. Chen, S. Liu, et al., Chemoresistance to FK228 is mediated by reversible MDR1 induction in human cancer cell lines, J. Pharmacol. Exp. Ther. 314 (2005) 467–475.

[15] Y. Sawakawa, Y. Naoe, T. Inoue, T. Sawakawa, M. Matsuo, T. Manda, et al., Effects of FK228, a novel HDAC inhibitor, on tumor growth and expression of p21 and c-myc genes in vivo, Cancer Lett. 195 (2003) 161–168.

[16] H. Sawa, H. Murakami, M. Kumagai, M. Nakasato, S. Yamauchi, N. Matsuyama, et al., HDAC inhibitor, FK228, induces apoptosis and suppresses cell proliferation of human glioblastoma cells in vitro and in vivo, Acta Neuropathol. 107 (2004) 523–531.

[17] M. Duxbury, H. Ito, M. Zinner, S. Ashley, E. Whang, Inhibition of Src Tyr-kinase impairs inherent and acquired gemcitabine resistance in human pancreatic adeno-carcinoma cells, Clin. Cancer Res. 10 (2004) 2307–2318.

[18] M. Yezhelyev, G. Koehl, M. Guba, T. Brabletz, K.W. Jauch, A. Ryan, et al., Inhibition of Src Tyr-kinase as treatment for human pancreatic cancer growing orthotopically in nude mice, Clin. Cancer Res. 10 (2004) 8028–8036.

[19] R. Bagheri-Yarmand, R. Vadlamudi, R.A. Wang, J. Mendelsohn, R. Kumar, VEGF up-regulation via PAK1 signaling regulates heregulin-beta1-mediated angiogenesis, J. Biol. Chem. 275 (2000) 39451–39457.

[20] W. Kiosses, J. Hood, S. Yang, M. Gerritsen, D. Cheresh, N. Alderson, et al., A dominant negative PAK1 peptide inhibits angiogenesis, Circ. Res. 90 (2002) 697–702.
[21] T. Nheu, H. He, Y. Hirokawa, K. Tamaki, A.W. Burgess, L. Florin, et al., The K252a derivatives, inhibitors for the PAK/MLK kinase family, selectively block the growth of RAS transformants, Cancer J. 8 (2002) 328–336.

References (supplement)

[22] A.P. Krapcho, C.S. Kuell, Synth. Commun. 20 (16) (1990) 2559–2564.
[23] T. Sifferlen, M. Rueping, K. Gademann, B. Jaun, D. Seebach, Helv. Chim. Acta 82 (1999) 2067–2093.
[24] US Patent 02, 749, 353.