Therapeutic Effects of an Anti-Myc Drug on Mouse Pancreatic Cancer

Dimitris Stellas, Matthias Szabolcs, Sanjay Koul, Zhe Li, Alexander Polyzos, Constantinos Anagnostopoulos, Zoe Cournia, Constantin Tamvakopoulos, Apostolos Klinakis, Argiris Efstratiadis

Manuscript received January 13, 2014; revised May 31, 2014; accepted August 21, 2014.

Correspondence to: Argiris Efstratiadis, MD, PhD, Biomedical Research Foundation, Academy of Athens, 4 Soranou Ephesiou, Athens 11527, Greece (e-mail: arg@bioacademy.gr).

Background Pancreatic ductal adenocarcinoma (PDA) is frequently driven by oncogenic KRAS(KRAS*) mutations. We developed a mouse model of KRAS*-induced PDA and, based on genetic results demonstrating that KRAS* tumorigenicity depends on Myc activity, we evaluated the therapeutic potential of an orally administered anti-Myc drug.

Methods We tested the efficacy of Mycro3, a small-molecule inhibitor of Myc-Max dimerization, in the treatment of mouse PDA (n = 9) and also of xenografts of human pancreatic cancer cell lines (NOD/SCID mice, n = 3–12). Tumour responses to the drug were evaluated by PET/CT imaging, and histological, immunohistochemical, molecular and microarray analyses. The Student’s t test was used for differences between groups. All statistical tests were two-sided.

Results Transgenic overexpression of KRAS* in the pancreas resulted in pancreatic intraepithelial neoplasia in two-week old mice, which developed invasive PDA a week later and became moribund at one month. However, this aggressive form of pancreatic tumorigenesis was effectively prevented by genetic ablation of Myc specifically in the pancreas. We then treated moribund, PDA-bearing mice daily with the Mycro3 Myc-inhibitor. The mice survived until killed at two months. PET/CT image analysis (n = 5) demonstrated marked shrinkage of PDA, while immunohistochemical analyses showed an increase in cancer cell apoptosis and reduction in cell proliferation (treated/untreated proliferation index ratio: 0.29, P < .001, n = 3, each group). Tumor growth was also drastically attenuated in Mycro3-treated NOD/SCID mice (n = 12) carrying orthotopic or heterotopic xenografts of human pancreatic cancer cells (eg, mean tumor weight ± SD of treated heterotopic xenografts vs vehicle-treated controls: 15.2 ± 5.8 mg vs 230.2 ± 43.9 mg, P < .001).

Conclusion These results provide strong justification for eventual clinical evaluation of anti-Myc drugs as potential chemotherapeutic agents for the treatment of PDA.


Mouse models faithfully simulating human cancer are valuable for genetic identification of potential drug targets but, among them, the most advantageous for practical use in subsequent preclinical testing of candidate therapeutic regimes are those exhibiting rapid tumor development. Considering that a KRAS mutation (predominantly in codon 12, such as KRASG12D, KRAS*) occurs with high frequency (about 90%) in cases of human pancreatic ductal adenocarcinoma (PDA) (1), we developed a mouse model of pancreatic tumorigenesis that is rapidly induced by oncogenic KRAS (KRAS*) and is suitable for preclinical studies.

To generate mouse tumors at chosen anatomical sites, we have used a variant of a genetic scheme based on Cre/loxP recombination (2,3). Depending on the tissue-specificity of the promoter driving Cre expression, corresponding tumors develop in progeny derived from matings between Cre-producer mice and Cre-responder partners carrying a dormant oncogenic transgene (usually in cDNA form), which becomes functional after excision of an upstream-positioned DNA segment flanked by loxP sites (“floxed”) that blocks its expression. As recipient sites for transgenic knock-in of various sequences, including a constitutively active oncogenic KRAS cDNA (KRASG12D, KRAS*), we used either one of two highly expressed loci targeted by corresponding constructs (“cassettes”) (Supplementary Figure 1, available online): the Actb locus encoding cytoplasmic β-actin (2) and the Eef1a1 locus encoding a translation elongation factor (3).

In a previous application of this approach, we developed a model in which overexpression of KRAS* was placed into the Eef1a1 locus (KRAS*E) and activated by Cre driven by the promoter of the Wap gene (encoding a milk protein) was unexpectedly able to elicit in a single-step the appearance of invasive mouse mammary carcinomas with a median time of tumor-free survival (T_{95}) of only nine days (3). The important advantage of such short tumor-lateness was...
subsequently demonstrated in a successful preclinical drug trial (3). Encouraged by these results, we generated an analogous model of rapidly developing pancreatic cancer, considering that the human disease continues to be a vexing and unresolved problem in terms of clinical management (in most patients, this lethal malignancy has already metastasized and is resistant to treatment at the time of diagnosis, and the five-year survival rate is only about 6%) (4).

The first among several successful mouse models of PDA (reviewed in 5–7) was the outcome of pioneering work by Tuveson, Hingorani, and collaborators (8,9), who used a blocked “Lox–Stop–Lox (LSL)-KRAS” allele generated by modification of the endogenous locus (10). LSL-KRAS+ could be conditionally activated in the pancreas of bitransgenic mice by crossing with Pdx1-Cre producers (Pdx1 expression commences in pancreatic progenitor cells at embryonic day 8.5). Whereas noninvasive lesions (pancreatic intraepithelial neoplasia [PanIN]) started appearing in Pdx1-cre/LSL-KRAS+ mice at about two months of age and progressively exhibited increasing histological abnormalities ranging from low- (PanIN1A/B) to intermediate-grade (PanIN2) and then to high-grade in situ carcinoma (PanIN3), manifestation of invasive PDA occurred after the age of seven months with an incidence not exceeding approximately 20% in a period of one year (8,11). However, by addition of a conditionally expressed gain-of-function mutant form of the p53 tumor suppressor in tritransgenic Pdx1-cre/LSL-KRAS+/p53R172H mice (KPC model), the PDA incidence became 100% and the tumor latency was substantially reduced (T<sub>50</sub> about 5 months) (9). Analogous shorter latencies were observed by combining Pdx1-cre/LSL-KRAS+ with other genetic alterations (5–7).

Methods

Mice

Knock-in KRAS*E transgenic mice conditionally expressing an oncogenic KRAS<sup>4B</sup> cDNA (12) placed in the Actb locus (Supplementary Figure 1A, available online) were derived from male chimeras generated by standard procedures using targeted W9.5 ES cells. The KRAS*E mice (carrying the same KRAS* cDNA, but in the Eef1a1 locus) (Supplementary Figure 1B, available online) (3), the Wap-Cre mouse strain (13), and the mice carrying floxed Myc alleles (Myc<sup>eR</sup>) (14) have been described. The Pdx1-Cre mice (strain number 01XL5) (8) and the Pbn-Cre mice (PB-Cre4, strain number 01XF5) (15) were obtained from the National Cancer Institute’s Mouse Repository. Progeny carrying combinations of genetic modifications to derive cohorts of experimental animals were generated by standard breeding schemes. Animals were housed in individually ventilated cages in the Animal House Facility of the Biomedical Research Foundation, Academy of Athens under pathogen-free conditions, in full compliance with the recommendations of the Federation of Laboratory Animal Science Association. All procedures were approved by the institutional Biosafety Committee and the Greek Ministry of Agriculture on the basis of the European Directive 86/609 concerning the protection of animals used for experimental purposes.

Pharmacokinetic Analysis and Drug Treatment

Mycro3 (16,17) (chemical name 1H-pyrazole-4-carboxylic acid, 5-[[7-(chlorodifluoromethyl)-5-(2-furyl)pyrazolo[1,5-a]pyrimidin-2-yl]carbonyl]amino]-1-phenyl-, ethyl ester; also known as Ethyl 5-[[7-(chloro(difluoro)methyl)-5-(2-furyl) pyrazolo[1,5-a]pyrimidin-2-yl]carbonyl]amino]-1-phenyl-1H-pyrazole-4-carboxylate; MW 526.9 Da) was purchased from the Chemical Diversity Research Institute (www.ChemDiv.com). Instead of using standard intraperitoneal injections, we administered the water-insoluble Mycro3 to mice by oral gavage as an emulsion formed by addition of solid compound to an aqueous solution of 0.5% carboxymethylcellulose and subsequent sonication for 30 minutes. At the highest dose tested for pharmacokinetic analysis and efficacy studies (100 mg/kg body weight), the drug was well tolerated. To assess the pharmacokinetic behavior of Mycro3 following this dosing regimen, a tail-bleeding protocol was used for each experimental animal for serial collection of blood samples at selected time points (Supplementary Figure 2, available online). Each blood sample (5 µL) was added to 45 µL of 0.1 M citric acid, pH 4.5, and stored at -80 °C until processing. The blood concentrations of Mycro3 were determined by mass spectrometry (LC-MS/MS) using a modification of a described procedure (18) adapted for measurements of Mycro3 in blood samples. The drug reached a maximum average blood concentration of 427 ng/mL (0.8 µM) at four hours post-dosing with an area under the curve (AUC) of one to 24 hours of 5449 ng.h/mL. Because a relatively high level (0.5 µM) was sustained for up to 12 hours, Mycro3 was administered for efficacy studies once daily for time periods indicated in the main text. Treatment results were monitored by PET/CT for PDA and by palpation for mammary tumors.

Statistical Analyses

All variables in statistical analyses were continuous. Values were expressed as mean ± SD. Results were considered statistically significant if P was less than .05. Differences between groups were examined using the two-sided Student’s t test. Kaplan-Meier tumor-free survival curves were plotted and compared pairwise by the Mantel-Cox Log-rank test using StatView software. For animal studies (blind randomization), calculations showed that the power of each test used for comparison was greater than 80%.

For details of cell culture experiments, xenograft studies, PET/CT imaging, and histological, immunohistochemical, molecular, and microarray analyses, see the Supplementary Methods (available online).

Results

Rapid Induction of PDA by Transgenic Overexpression of Oncogenic KRAS

To develop our mouse PDA model, we followed a key feature of a reported approach (8) and used Pdx1-Cre producer mice. However, instead of a mutated endogenous locus presumably transcribing KRAS* at physiological levels, our Cre-responder animals carried a blocked KRAS* cDNA placed into our “Actb cassette” (KRAS*4) for ectopic overexpression (unexpectedly, the “Eef1a1 cassette” turned out to be unsuitable for our purposes in this case, as the inserted KRAS*E resulted in the appearance of pancreaticoblastoma-like tumors in mouse embryos).
Whereas control animals with either a \textit{KRAS*}A or a \textit{Pdx1-Cre} genotype did not develop tumors during a one-year period of observation (Figure 1A), we consistently observed that between two and three weeks after birth the bitransgenic \textit{Pdx1-cre/KRAS*}A mice started exhibiting signs of a bloated abdomen, became increasingly sluggish and cachectic, and were moribund by the first month of age (only 2 of 42 mice were able to survive for 45 days) (Figure 1A).

We attribute this rapid development of PDA to the high levels of transgenic \textit{KRAS*} expression, previously noted to parallel its potency in tumorigenicity (19). Using quantitative–reverse transcription–polymerase chain reaction (Q-RT-PCR), we found that the level of total \textit{KRAS} transcripts in \textit{Pdx1-Cre/KRAS*}A-induced PDA (n = 4) was 29.4±13.2-fold higher than normal (n = 3), while the corresponding ratio of \textit{KRAS} protein determined by western analysis was 28.5±6.9 (n = 5) (Figure 2A). Upon necropsy, gross examination showed that the entire pancreas of our mice had the appearance of a solid tumor. Histopathological analysis of pancreata harvested at an early postnatal age (day p7, n = 6) did not reveal abnormalities, whereas 1 of 2 mice examined at p10 exhibited PanIN2 (Figure 1B). At p14, multifocal atypical acinar-to-ductal metaplasia was observed together with PanIN3 consisting of ducts lined by atypical cells that formed tufts and papillary structures, while early invasion was also noted (2 of 2 mice) (Figure 1B). In sections from p15 and older mice examined at almost daily intervals we observed, in addition to PanIN3, development of invasive PDA, which from p19 onward had replaced virtually all of the normal pancreatic parenchyma. This adenocarcinoma was moderately differentiated and exhibited preserved, albeit irregular glandular structures that had elicited a marked desmoplastic response in the surrounding inflamed stroma (Figure 1B). Metastases were not observed, apparently because of the early time of mouse death.

Although the results of the histopathological analysis of the \textit{Pdx1-cre/KRAS*}A neoplasms were the same as those reported for \textit{Pdx1-Cre/LSL-KRAS*} tumors (8,9), the extreme difference in latencies was a matter of concern, considering that the phenotypic manifestations caused by expression of endogenous activated oncogenes from their own promoters can differ from those resulting from ectopic transgenic overexpression (see, for example, Ref. 20).

Therefore, as an additional criterion that would support the recognition of the \textit{Pdx1-cre/KRAS*}A mouse strain as a bona fide model suitable for preclinical studies, we performed pairwise comparisons of microarray RNA profiles between \textit{Pdx1-cre/KRAS*}A PDA data and corresponding results from \textit{Pdx1-cre/LSL-KRAS*}A mice (11) and also the transcriptomes (three independent datasets) of human PDA (21–23). In all of these comparisons, the results showed a high degree of statistically significant overlap in the expression of both upregulated and downregulated genes (Supplementary Table 1, available online).

\textbf{Myc as an Effector of KRAS*-Induced PDA}

Considering that many attempts over a long period of time failed to show that \textit{KRAS*} itself is “druggable,” we used our PDA mouse model to examine genetically whether Myc, a presumptive participant in the carcinogenic pathway acting downstream of \textit{KRAS*} (24), could be a potential drug target. Until recently, Myc was also considered “undruggable” because it was thought that systemic inhibition of its central transcriptional role affecting many functions would have devastating side effects. This view is now changing on the basis of results with Omomyc, a mutant bHLHZip Myc domain of 90 amino acids, which acts in a dominant-negative fashion as a competitive inhibitor of Myc/Max dimerization-dependent transcription by heterodimerizing with either Myc or Max (25). Systemic inhibition of Myc in mice carrying a conditionally activatable Omomyc construct resulted in mild and tolerable side effects, while Omomyc action in the lung caused regression of \textit{LSL-KRAS*}-induced non–small-cell adenocarcinomas (25).

The potential involvement of endogenous Myc in \textit{KRAS*}-driven mouse PDA appeared to be consistent with the following circumstantial evidence. Myc expression was found to be two-fold upregulated over normal in both the \textit{Pdx1-cre/LSL-KRAS*} (11) and our \textit{Pdx1-cre/KRAS*}A microarrays, while the corresponding ratio determined in our case by Q-RT-PCR was 3.7±2.4 (n = 3). Such an increase at the transcriptional level was not observed in the RNA profiles of human PDA (21–23), but immunohistochemical (IHC) analyses (26–28) have shown positive staining for Myc protein in a large fraction of human PanIN and PDA specimens (40% to 70%), in contrast with normal pancreata (absence of immunoreactivity).

Similarly, we observed very high expression of Myc protein both by IHC and Western analyses in PDAs of our \textit{Pdx1-cre/KRAS*}A model, whereas Myc was practically undetectable by these assays in normal pancreata (Figures 1C and 2A). We also found high expression of phospho-Erk1/2 (pErk) and phosphoAkt1 (pAkt1) in PDA by IHC, in contrast with the control pancreata (negative results) (Figure 1C), consistent with the view that high \textit{KRAS*} signaling stabilizes Myc (29) through activation of the Raf-MEK-ERK pathway, inducing Myc phosphorylation at S62, and concomitant activation of the PI3-kinase-Akt1 pathway that inhibits GSK3b, thus suppressing the Myc degradation-promoting T58 phosphorylation. Moreover, the RNA profile of our mouse model was statistically significantly enriched not only for previously reported \textit{KRAS*}-induced genes and signatures, as expected, but also for Myc-induced transcripts and Myc target genes (Supplementary Tables 2–4, available online). Interestingly, it was recently shown that conditional expression of a \textit{Myc} transgene in the pancreas largely phenocopies the effects of \textit{KRAS*}, leading to the development of PDA with a T\textsubscript{\textit{50}} of approximately 80 days (28).

To ascertain the potential involvement of Myc in pancreatic carcinogenesis, we used a cohort of 25 tritransgenic mice with a \textit{Pdx1-cre/KRAS*}A/\textit{Myc\textsuperscript{\textit{d00}}} genotype, in which the floxed alleles of the endogenous \textit{Myc} locus (14) could be conditionally ablated at the time of activation of \textit{KRAS*} expression. We killed these mice at various times ranging from about three weeks to many months for histopathological evaluation of the consequences of Myc ablation (two of the mice died of natural causes at ages >2.5 years) (Figure 1A). Not unexpectedly, acinar atrophy to various degrees, but with preservation of the ductal and endocrine compartments, was generally observed in bitransgenic \textit{Pdx1-cre/Myc\textsuperscript{\textit{d00}}} control mice (not shown), as reported (30). In tritransgenic \textit{Pdx1-cre/KRAS*}A/\textit{Myc\textsuperscript{\textit{d00}}} mice, however, atrophy was a rare occurrence (Figure 1D), while none of the mice exhibited invasive pancreatic cancer (only PanIN1B-3 was found in some mice older than seven months) (Figure 1D). While this work was in progress, it was reported that two mice with a \textit{Pdx1-cre/LSL-KRAS*}A/\textit{Myc\textsuperscript{\textit{d00}}} genotype developed PanIN1 but not PDA at 11 and 12 months of age (31).
Figure 1. Myc-dependence of oncogenic KRAS (KRAS*)–induced pancreatic cancer. **A)** Mice of the indicated genotypes were killed at various time points (small vertical lines) for histopathological analyses to determine the phenotypes shown. On occasion, more than one animal (2–6) was killed at the same time (indicated by numbers beneath particular vertical lines). The number (n) of animals in each cohort is shown on the left of the Figure. The data cannot be presented in the format of a Kaplan-Meier survival curve because the mice were not moribund at the time of euthanasia, with the exception of 30-day old (or older) mice with a **Pdx1-Cre/KRAS*A** genotype. For a detailed display, the data pertaining to the extremely rapid appearance of pancreatic ductal adenocarcinoma (PDA) in **Pdx1-Cre/KRAS*A** mice are also shown in a blow up (time scale in days, rather than in months) at the top of the Figure. In the absence of Myc (**Pdx1-Cre/Myc*fl/fl** genotype), the carcinogenic action of KRAS* is not manifested, even after 2.5 years. **B)** Histological sections of pancreata harvested from **Pdx1-Cre/KRAS*+** animals at the times shown document the rapid appearance of KRAS*-induced PanIN and PDA within the first three postnatal weeks (hematoxylin/eosin staining). **C)** Myc is practically undetectable by immunostaining in normal pancreas, whereas its expression in PDA is very high (see also Figure 2A). Similarly, pErk and pAkt1 are highly expressed in KRAS*-induced PDA, in contrast to normal pancreas. **D)** In the absence of Myc in mice with a **Pdx1-Cre/KRAS*/Myc*fl/fl** genotype, KRAS* is unable to induce PDA and the pancreatic parenchyma remains normal or occasionally exhibits PanIN in mice older than seven months of age (immunostaining and H&E staining). Cre-mediated Myc ablation appears to be complete, except for rare PanIN foci (weak nuclear Myc immunoreactivity). Rarely, there is evidence of atrophy, in contrast with the frequent incidence of atrophic pancreas in **Pdx1-Cre/Myc*fl/fl** mice lacking oncogenic KRAS*. **Scale bars** in **B**, **C**, and **D**: 50 µm, except for the first panel in **D** (200 µm).
To expand our observations, we also showed that genetic ablation of Myc rescues the mice from the development of KRAS*-induced mammary and prostatic adenocarcinoma and penile squamous cell carcinoma (Supplementary Results and Supplementary Figures 3 and 4, available online). We conclude, therefore, that our results in conjunction with the Omomyc data (25) unequivocally demonstrate that the functions of endogenous Myc are indispensable for the tumorigenic action of KRAS* in a variety of carcinomas.

Treatment of KRAS*-Induced Carcinomas by Oral Administration of an Anti-Myc Drug

The causal KRAS*/Myc relationship, which was established by genetic intervention and can be viewed as an analog of “chemoprevention,” provided ample justification for a preclinical “chemotherapy” trial using known small-molecule Myc inhibitors as candidate drugs, despite the fact that such previous efforts failed (32). In this regard, we first evaluated in vitro compounds blocking Myc/Max dimerization (17) by using two cell lines of common origin, TGR-1 (Myc+/+) and H015.19 (Myc−/−) (33). We observed that one of the tested molecules, Mycro3 (16,17), exhibited in this assay an excellent window of specificity (IC50 of 0.25 vs 9.0 µM for cells with intact Myc alleles and Myc-null cells, respectively) (Supplementary Figure 5, available online). The higher sensitivity of TGR-1 cells to Mycro3 in comparison with other cell lines, such as U-2OS osteosarcoma cells (IC50 of 10 µM) (16), is not surprising, as the strength of Mycro3 activity is cell line–dependent (34).

Importantly, in contrast to another potent Myc inhibitor, 10058-F4, which lacks antitumor activity in vivo apparently because of
poor pharmacokinetic behavior (35), the results of our pharmacokinetic analysis for Mycro3 were satisfactory (Supplementary Figure 2, available online). Thus, we found that Mycro3, after reaching a maximum blood concentration of 0.8 μM at four hours post-dosing, is sustained in the mouse circulation for at least 12 hours at concentrations adequate for efficacy studies (0.5 μM). We also found that, although water-insoluble, Mycro3 can be administered to mice by oral gavage as an emulsion (see Methods).

To examine the potential “true” chemotherapeutic effect of Mycro3, we started all treatments at the time that practically all Pdx1-cre/KRASG12D mice became moribund because of full-fledged PDA and required killing (about 30 days). Thus, the experimental group of mice (n = 9), which were treated daily by oral administration of Mycro3 at a dose of 100mg/kg for two months and then killed for analysis, could not be compared with a control group receiving only vehicle. Nevertheless, the long-term survival of the originally moribund experimental animals was indicative of a successful treatment, especially considering that in one case, in which Mycro3 administration was discontinued after two months, the mouse survived for an additional month before being killed for analysis. Overt toxic effects of Mycro3 were not noticed. The efficacy of Mycro3 was further demonstrated, following 18F-Fludeoxyglucose (18F-FDG) injection, by PET/CT image analysis before and after treatment (n = 5), which showed marked shrinkage of tumors (Figure 2B), and by the results of histopathological analysis, which revealed tissue scarring and presence of normal acini, although remnants of cancer cells positive for cytokeratin-19 (CK19) immunostaining were also evident (CK19 colocalizes with KRAS in PDA) (Figure 2, C and D). IHC analysis using antibodies against Ki-67 and caspase-3 showed, respectively, reduction in cell proliferation and increase in apoptosis (Figure 2C). The Ki-67-assessed proliferation indices (PI) of treated vs untreated PDA specimens (n = 3, each) were 22.5 ± 3.3% and 76.4 ± 2.8%, respectively (P < .001, treated/untreated PI ratio = 0.29). In excellent agreement with these measurements, postmortem (after imaging) gamma counting of the 18F-FDG radioactivity indicated that the uptake of the label in treated pancreatic specimens was 29.75 ± 5.0% of that in untreated PDA (n = 3 each). We conclude, therefore, that the average residual tumor after Mycro3 treatment is approximately 30%. We note for clarity that the untreated (control) tumors were harvested from 30-day-old moribund animals.

Verification that Mycro3 functions through Myc inhibition in PDA treatment was obtained from the analysis of microarray results comparing the transcriptomic profiles of untreated (control) and Mycro3-treated primary cultures of the epithelial component of mouse PDAs (the control cultured cells faithfully represent the tumor transcriptome) (see Supplementary Table 5, available online). The profile of downregulated genes in Mycro3-treated cells exhibited a statistically significant degree of overlap with genes also downregulated by the Myc inhibitor Omomyc (36), with genes that are normally upregulated by Myc (five different datasets), and with genes that are known direct Myc targets (five different lists) (Supplementary Tables 5 and 6, available online).

To evaluate Myc as a Mycro3 drug target in human cells, we treated mice carrying orthotopic and heterotopic xenografts generated in NOD/SCID mice by injection of the human PDA cell lines Panc1 (KRASG12D) and MiaPaCa2 (KRASG12C). We observed that tumor growth was drastically attenuated in Mycro3-treated mice in comparison with vehicle-treated controls. Thus, in treated mice, the weights of pancreatic specimens containing orthotopic xenografts were 46.0 ± 13% (Panc1; P = .012) and 37.8 ± 5.9% (MiaPaCa2, P = .019) of the control weights, while the corresponding values for the weights of cleanly excised heterotopic xenografts were 9.8 ± 1.7% (Panc1, P < .001) and 6.6 ± 2.5% (MiaPaCa2, P < .001) (eg, mean tumor weight ± SD of treated vs untreated heterotopic MiaPaCa2 xenografts: 15.2 ± 5.8 mg vs 230.2 ± 43.9 mg, P < .001) (Figure 3). Ki-67 immunostaining indicated that the proliferation of cancer cells in treated heterotopic MiaPaCa2 xenografts was markedly reduced in comparison with the controls (PI = 19.2 ± 1.0% and 51.0 ± 2.0%, respectively, P < .001, treated/untreated PI ratio = 0.38) (Figure 3). Mycro3 was also efficacious in treating xenografts generated either heterotopically or orthotopically (by tail injection) using the human lung carcinoma cell line A549 (KRASG12S), and also in treating mouse mammary tumors appearing in Wap-cre/KRASG12E mice (Supplementary Results and Supplementary Figures 3 and 6, available online).

Discussion

The potent anti-Myc therapeutic approach indicated by these results may contribute to the arsenal of targeted treatment options against various human cancers, including PDA. Currently, there is an imperative need for developing and evaluating in parallel novel regimen choices to manage advanced PDA considering that, despite numerous preclinical and clinical studies in recent years, the standard of care is merely the administration of gemcitabine, alone or in combination with other chemotherapeutics, which, unfortunately, has limited clinical benefit (median overall survival of five to eight months) (37).

Further preclinical evaluations of the efficacy of gemcitabine in novel combinations with other agents were recently performed using the KPC mouse model (reviewed in Ref. 38) and yielded promising, although modest, results (increase of survival time by about two to four weeks). The anti-Myc treatment outcome using our own model of aggressive mouse PDA (increase of survival time by at least two months) exemplifies the importance of attempting applications of alternative therapeutic schemes, which could increase the efficacy of combination regimens. These results, in conjunction with our treatment data using human PDA xenografts and other mouse cancer models, provide strong justification for detailed evaluations of Myc inhibitors, such as Mycro3 and derivatives generated by chemical modifications and optimized for efficacy, as candidate drugs that could be eventually tested in clinical trials as chemotherapeutic agents.

We note that we did not yet have the opportunity to make detailed long-term observations after discontinuing the Mycro3 treatment at various time points, a limitation of this preclinical study. Also, an apparent limitation of the therapeutic regime, not unexpected for a monotherapy protocol, is that the tumors of the treated mice, although markedly reduced in size in comparison with the untreated controls, do not completely disappear (attempts...
to assess the efficacy of Mycro3 in combination with other drugs are now in progress).

In conclusion, considering that Myc is frequently deregulated in a wide variety of human malignancies (39) and could serve as a common effector channeling the action of various converging carcinogenic pathways in different cancer types, it is not unlikely that anti-Myc drugs might become a key common component of otherwise variable combinatorial chemotherapeutic protocols.

Figure 3. Mycro3-treatment of xenografts generated by orthotopic or heterotopic transplantation of the human pancreatic ductal adenocarcinoma (PDA) cell lines Panc1 and MiaPaCa2. A) Administration of Mycro3 or vehicle started two weeks after xenotransplantation and lasted for two weeks. In the case of orthotopic xenografts, the effect of Mycro3, although statistically significant, is underestimated because recovery of exclusively tumor tissue was not possible (the weight of the entire tumor-bearing pancreas was determined at the end of the experiment). In contrast, each heterotopic tumor could be cleanly excised for weight determination. Data are means and error bars represent SD. P values by two-sided Student’s t test. B) Gross appearance of surgically recovered Mycro3-treated or untreated (control) heterotopic MiaPaCa2 xenografts. Scale bar = 1.0 cm. C) Mycro3 treatment did not change the histopathological appearance of xenografts, as the comparison of sections from treated and untreated specimens indicates (H&E staining). However, extensive necrosis was noted in the treated xenografts, along with marked reduction of proliferating cancer cells, as shown by Ki-67 immunostaining (n = 3 mice; four sections per mouse). Scale bars for all panels in C: 50 µm (if not shown, the bar length should be considered as equal to that in the first panel).

References


Funding
This study was supported by funds provided to the Biomedical Research Foundation, Academy of Athens by a Krauss Maffei Wegmann GmbH and Co Offset Program. Seed funding for a preliminary phase of the work was provided by a grant to AE from the Lustgarten Foundation (LF01-057).

Notes
D. Stellas designed experiments, performed the main experimental work, and analyzed data. M. Szabolcs performed the histopathological and immunohistochemical analyses and interpreted the data. S. Kouli and Z. Li contributed to the genetic studies. A. Polyzos provided bioinformatics support. C. Anagnostopoulos performed the PET/CT imaging study and analyzed the data. Z. Cournia contributed computer-aided analyses to select candidate anti-Myc drugs. C. Tannakopoulos performed the pharmacokinetic analyses. A. Klinakis and A. Efstratiadis conceived and designed the study. A. Klinakis contributed to the genetic studies and analyzed data. A. Efstratiadis supervised the project, analyzed the data, and wrote the paper.

We thank John Sedivy for kindly providing the TGR-1 and H015.19 cell lines, Kay-Uwe Wagner, Michael Cole, and John Sedivy for kindly making available detailed microarray data from their studies, and (in alphabetical order): A. Agapaki, I. Baira, S. Karaliota, N. Kostimtsopoulos, N. Koutsogiannis, D. Papadopoulou, and A. Varela for technical advice and/or assistance.

Affiliations of authors: Biomedical Research Foundation, Academy of Athens, Athens, Greece (DS, AP, CA, ZC, CT, AK, AE); Department of Pathology and Cell Biology (MS) and Division of Hematology/Oncology (SK), Columbia University Medical Center, New York, NY; Regeneron Research Laboratories, Tarrytown, NY (ZL).