Efficacy of PHA-848125, a Cyclin-Dependent Kinase Inhibitor, on the K-RasG12DLA2 Lung Adenocarcinoma Transgenic Mouse Model: Evaluation by Multimodality Imaging

Anna Degrassi1, Micaela Russo1, Cristina Nanni3, Veronica Patton1, Rachele Alzani1, Anna M. Giusti2, Stefano Fanti3, Marina Ciomei1, Enrico Pesenti1, and Gemma Texido1

Abstract

K-ras is the most frequently mutated oncogene in non–small cell lung cancer (NSCLC), the most common form of lung cancer. Recent studies indicate that NSCLC patients with mutant K-ras do not respond to epidermal growth factor receptor inhibitors. In the attempt to find alternative therapeutic regimes for such patients, we tested PHA-848125, an oral pan cyclin-dependent kinase inhibitor currently under evaluation in phase II clinical trial, on a transgenic mouse model, K-RasG12DLA2, which develops pulmonary cancerous lesions reminiscent of human lung adenocarcinomas. We used magnetic resonance imaging and positron emission tomography to follow longitudinally disease progression and evaluate therapeutic efficacy in this model. Treatment of K-RasG12DLA2 mice with 40 mg/kg twice daily for 10 days with PHA-848125 induced a significant tumor growth inhibition at the end of treatment ($P < 0.005$) and this was accompanied by a reduction in the cell membrane turnover, as seen by 11C-Choline-positron emission tomography ($P < 0.05$). Magnetic resonance imaging data were validated versus histology and the mechanism of action of the compound was verified by immunohistochemistry, using cyclin-dependent kinase–related biomarkers phospho-Retinoblastoma and cyclin A. In this study, multimodality imaging was successfully used for the preclinical assessment of PHA-848125 therapeutic efficacy on a lung adenocarcinoma mouse model. This compound induced a volumetric and metabolic anticancer effect and could represent a valid therapeutic approach for NSCLC patients with mutant K-ras.

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Introduction

Lung cancer is the leading cause of cancer deaths worldwide (1). The prevalence of adenocarcinoma, a form of non–small cell lung cancer (NSCLC), is increasing and is currently the most common form of lung cancer (2). Alteration of the major regulatory pathways either by gene overexpression or mutation is a frequent event in lung cancer. It is widely accepted that epidermal growth factor receptor (EGFR), K-Ras, and mitogen-activated protein kinase function sequentially in the EGFR signaling pathway and this pathway plays a fundamental role in NSCLC (3, 4). K-ras oncogene is frequently mutated in human tumors and activating mutations in K-ras occur in 30% of NSCLC.

Despite a growing understanding of the aberrant molecular mechanisms responsible for lung cancer onset, treatment of lung cancer has only marginally improved and still relies for the vast majority to the surgical procedure. Novel therapies, validated on new animal cancer models, which better recapitulate the human disease, are therefore urgently needed.

In the K-RasG12DLA2 transgenic mouse model, a latent mutated K-Ras allele is sporadically activated and, as a consequence, these mice develop lung adenocarcinomas (5). Because mutant gene expression is under its normal physiologic control and it occurs in scattered cells surrounded by normal cells, this model recapitulates spontaneous oncogene activation as seen in human cancer and may more accurately mimic the interaction of tumor cells with their environment. Moreover, a study comparing K-RasG12DLA2 tumors to human lung tumors revealed a molecular similarity of these mouse tumors to human lung adenocarcinoma (6).

New and more sophisticated animal models pose some difficulties in terms of lesion assessment and monitoring; imaging techniques, able to detect and monitor noninvasively the development and growth of malignancies, are needed in these models to evaluate the efficacy of novel therapeutic intervention (7).

Imaging methodologies are routinely used in the clinic for diagnosis and follow-up of lung malignancies. In recent years, preclinical imaging techniques such as magnetic resonance imaging, positron emission tomography, and optical imaging have shown potential for improving the understanding of tumor biology and for evaluating new therapeutic agents. In this study, we used multimodality imaging to assess the therapeutic efficacy of PHA-848125, an oral pan cyclin-dependent kinase inhibitor, in a K-RasG12DLA2 transgenic mouse model.
as small-animal magnetic resonance imaging (MRI) and small-animal positron emission tomography (PET; refs. 8, 9) have been used in oncology research for in vivo evaluation of tumors and to assess response to therapy (10–13).

MRI provides very high resolution and excellent soft tissue contrast but it has not traditionally been used for the visualization and study of lung pathology because this organ presents a unique challenge for MRI. A review of small-animal MRI of lung is presented in Schuster et al. (14). Reliable methods for respiratory and cardiac gating are now routinely available and the extremely low signal of healthy lung parenchyma facilitates the detection of lung tumors by providing a dark background against which pathologic lesions can be identified. Recently, two- and three-dimensional MRI, using both spin echo and gradient echo sequences, were used to detect and follow mouse pulmonary tumors in various lung cancer models. Investigators used animals injected intratracheally with human lung carcinoma cells (15), mice treated with the carcinogen benzopyrene (16) or urethane (17), and transgenic models (18, 19).

The good sensitivity and spatial resolution (around 1.0–1.5 mm) offered by the new small-animal PET tomographs (20) allow an in vivo measurement of the metabolic activity of neoplastic masses, this being a crucial issue in the evaluation of therapy efficacy. A PET scan is able to estimate the expression of different metabolic patterns within the tumor by using the most appropriate tracer: 18F-FDG highlights cellular glucose consumption, 11C-methione highlights cellular protein synthesis, whereas 11C-Choline (Cho) is an indicator of cellular membrane turnover.

Recent studies indicate that NSCLC patients with mutant K-Ras tumors do not respond to EGFR inhibitors (21, 22), and this agrees with K-Ras being a downstream effector of EGFR. Thus far, no direct Ras inhibitors have proven being clinically effective; therefore, the development of agents aimed to inhibit pathways downstream of mutant K-Ras tumors do not respond to EGFR inhibitors PHA-848125 is a novel CDK inhibitor that just entered phase II clinical development (25) as an oral anticancer treatment for patients with advanced malignancies. It is a CDK2, CDK1, CDK4, and TRKA inhibitor belonging to the pyrazol[4,3-h]quinazoline chemical class (26). We decided to investigate whether PHA-848125 exerts its antitumor efficacy also in the K-Ras<sup>G12D</sup>L2 transgenic model of lung cancer.

Here, we present our work in which two imaging methodologies, such as small-animal MRI and 11C-Cho PET, were used to fully characterize the K-Ras<sup>G12D</sup>L2 transgenic mice and to test the efficacy of the CDK inhibitor PHA-848125. Histology was done to characterize pulmonary lesions and validate imaging data. The mechanism of action of our compound was evaluated by immunohistochemistry, by looking at target modulation in the CDK pathway.

Materials and Methods

Animals and Efficacy Studies

All procedures adopted for housing and handling the animals were in strict compliance with the European Communities Council and Italian Guidelines for Laboratory Animal Welfare.

K-Ras<sup>G12D</sup>L2 mice were obtained from the Massachusetts Institute of Technology and bred in our facilities. The K-Ras<sup>G12D</sup>L2 mice used were in 129B6F1 genetic background and were genotyped as described elsewhere (5).

For the model characterization study, a large cohort of animals at different ages, ranging from 10 to 30 wk, underwent serial MRI.

For the MRI efficacy study, eight control and eleven treated mice were imaged at day 0 (pretreatment), day 11, 21, and 32 for CDK-125–treated mice and at day 0, 11, 17, 24, and 43 for control animals.

For the PET efficacy study, six control and five treated mice underwent imaging at day 0 (pretreatment), day 5 and day 12 for the treated group, and at day 0 and day 12 for control animals.

The criteria for selecting the animals for the imaging efficacy studies were the following: age ranging between 10 and 20 wk and number of lesions between 4 and 10, with at least 2 measurable lesions. PHA-848125 was administered orally for 10 d at a dose of 40 mg/kg twice daily.

The immunohistochemistry study was done in five controls and five treated mice, which underwent the same treatment schedule.

Magnetic Resonance Imaging

A Bruker Pharmascan instrument operating at 7.0 T was used. Anesthetized animals (2–3% isoflurane gas with 0.5 L/min air) were positioned prone in the animal bed and inserted in the radiofrequency coil (38 mm internal diameter) inside the magnet. Electrodes for
electrocardiogram (ECG) monitoring and pneumatic sensor for respiratory monitoring were positioned on the mouse. Scout transverse images were acquired for correct positioning of thoracic pulmonary region. A spin echo sequence (Bruker MSME sequence: field of view, 4 × 4 cm; matrix, 256 × 128; spatial resolution, 156 μm; repetition time, 1,000 ms; effective echo time, 12 ms; four averages; Resp and ECG triggering) was used for morphologic examination and tumor volume measurements. Twenty-two adjacent 0.55-mm-thick coronal slices were acquired all across the lung area. The whole acquisition, including induction of anesthesia, positioning, and set up, took ∼20 min per animal. A macro was used to calculate tumor volumes (in mm³) from the area of all slices covering the tumor and their slice thickness. For therapy efficacy evaluation, the volume of two or three lesions per animal was measured. % growth [(tumor volume day X – tumor volume day 0) × 100/tumor volume day 0] of considered lesions were first combined and averaged for each animal; an average for all mice was then calculated and plotted for control and treated groups, with SDs calculated from the average % growth on a per animal basis. Efficacy evaluation was also done according to the Response Evaluation Criteria in Solid Tumors criteria used in the clinic (27).

Positron Emission Tomography

The animals that underwent PET imaging were first selected by MRI to ensure the presence of at least two significant pulmonary masses.

The whole diagnostic procedure was carried out on all animals in similar metabolic conditions and under a warm light to maintain the body temperature.

A small-animal PET tomograph (GE, eXplore Vista DR) was used and 11C-Cho was used as a tracer. Anaesthetized animals (3–5% sevofluorane and 1 L/min oxygen) were injected in the tail vein with 0.1 mL of 20 MBq of 11C-Cho and placed prone on the scanner bed; after an uptake of 5 min, images were acquired for 15 min (one bed position; field of view, 4 cm).

11C-Cho PET images were reconstructed iteratively (OSEM 2D) and read in three planes. The scan was considered positive if at least one area of increased Cho uptake was present in the lungs. Semiquantitative analysis was carried out for each identified tumor using the target to background ratio (TBR). As common practice in clinical PET, the target region of interest (ROI) was placed on the most active area of the neoplastic mass and the background ROI was placed in the s.c. tissue of the interscapular region. TBR was finally calculated as max count in the target ROI/mean count in the background ROI.

The TBR of the most active masses at different time points were compared in control and treated animals to evaluate therapy efficacy.

Histology and Immunohistochemistry

Mice were euthanized using carbon dioxide inhalation, and the lungs were collected. For the mechanism of ac-
acquired at day 0 and day 105: tumor lesions detected at day 0 increased in size at a later time and new lesions also became detectable. The relatively long time frame of disease progression in this model had to be taken into account for planning the efficacy study.

The graph in Fig. 1C indicates the frequency of lesions in analyzed mice according to age: at 10 to 12 weeks of age only a small percentage of the animals examined presented a number of lesions higher than 10, whereas this percentage dramatically increased in mice ages 26 to 30 weeks. At the same time, old animals very rarely showed no or few lesions, with this feature being the predominant pattern in 10- to 12-week-old K-RasG12DLA2 mice.

Acidophilic macrophage pneumonia, observed histologically in some animals, had to be taken into account to avoid an erroneous interpretation of MR images and the inclusion of nonneoplastic lesions when measuring disease progression or response to therapy. Inflammatory regions showed a patchy pattern on MRI, rarely circular in shape, which often involved a large if not the whole lobar area (Fig. 1D). Therefore, when carefully examined, these areas could be quite easily excluded by the analysis.

By looking at lesion incidence and growth in time, we decided to select and insert in the efficacy study mice with an age ranging from 10 to 20 weeks and presenting a number of lesions between 4 and 10, with at least 2 measurable lesions (≥1 mm diameter). The idea behind this choice was to select a reasonably homogeneous group of animals, by neglecting very old animals and those showing a too early or too advanced stage of the disease. Furthermore, bigger lesions were reasonably less prone to volume measurement errors.

Figure 2A shows the results obtained for the PHA-848125 inhibitor study. Images are shown for a control and a treated mouse at day 0 and day 11 (1 day posttreatment): whereas tumor volume is slightly increased in the control animal, a slight decrease in lesion size can be appreciated in the animal that underwent the treatment. The graph in Fig. 2B shows the % tumor growth curve for control and treated groups: average values are reported with error bars indicating SDs. A significant (P < 0.005, Student’s t test) decrease in tumor growth was observed 1 day after the end of treatment in the group receiving the CDK inhibitor. After the end of treatment cycle, a slow regrowth of treated tumors was observed. We noticed a decreased number of lesions in treated animals when compared with controls, but this difference was not significant; tumors, in fact, clearly decreased in size but often remained detectable.

In the general attempt to perform reliable preclinical trials, whose results could be easily translated to the clinic, we also examined our MRI data according to the Response Evaluation Criteria in Solid Tumors evaluation, which dictates the guidelines used by clinicians to score response in solid tumors (27). According to such evaluation criteria, partial response (PR) and progressive disease (PD) are assigned to those subjects with a target lesion that shows, respectively, at least a 30% decrease (PR) and 20% increase (PD) in the sum of the longest...
diameters; whether there is neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, the state of stable disease is assigned.

We considered two target lesions per animal: at the end of the treatment with our inhibitor, 8 of 11 K-RasG12DLA2 mice showed a PR, whereas the remaining 3 treated animals showed a stable disease; in the control group, 4 mice of 8 showed a stable disease, whereas for the remaining 4 control mice, a state of PD was assigned.

**PET Imaging**

Figure 3 shows Cho-PET images of a control (A) and a treated (B) animal. When comparing the hypermetabolic pulmonary masses in the control and treated animal, we can clearly appreciate how the mass remains stable in the control animal, whereas it disappears at the end of the PHA-848125 therapy cycle.

Because TBR is an index of the metabolic activity of the tumor, TBR of the most active mass in all mice at different time points are reported in Table 1. Treated animals present a neoplastic disease that shows a significant reduction in metabolic activity after completion of therapy. Controls, on the other hand, present a metabolic index that either increases over time or remains stable: the decrease in TBR measured in some animals is in fact only just above 25%, which is the European Organization for Research and Treatment of Cancer significance level, and therefore classifies as stable disease. Mean values of TBR for control and treated animals, together with SD values, are also reported in Table 1. A significant decrease ($P < 0.05$, Student’s $t$ test) in the mean TBR was obtained at the end of the treatment with the CDK inhibitor.

**Histology and Immunohistochemistry**

Histology was done in mice at different ages for model characterization; furthermore, gross examination and histologic analysis of lung lesions was used to validate the MRI data.

For histologic characterization of the model, mice as young as 4 weeks were used. Nodular lesions were detected in all transgenic mice considered. Proliferative lesions included hyperplasia, benign, and malignant tumors. The most frequently recorded lesions were adenomas; malignant lesions were generally solid adenocarcinomas, with fewer microcarcinomas (Fig. 4C–E). The size of the lesions was not indicative of their nature: lesions of the same size could have histologic features of adenoma or adenocarcinoma. Tumor burden progressively increased with age, with the number of lesions increasing from an average of 11 lesions in a 4-week-old animal to >50 in a 20-week-old mouse. Tumors were always multiple, with early, well-differentiated lesions, and more advanced malignant lesions coexisting within the same animal, suggesting an asynchronous tumor development. Acidophilic macrophage pneumonia was observed in some animals; this unusual pneumonia was occasionally reported in some mouse strains, including the 129sv strain (29).

Figure 2. MRI efficacy study. A, representative MR images in a control (top) and treated (bottom) animal pretreatment (day 0) and posttreatment (day 11). B, % tumor growth for control and treated groups; points, mean; values with error bars, SD.

Figure 3. $^{11}$C-Cho PET efficacy study. Cho-PET images of a treated (A) and control (B) mice.
To validate the MRI data, a high resolution histologic slice of the whole lung was reconstructed for five samples and count of lesions was compared with the *in vivo* imaging data. Figure 4A shows the histologic slice and the corresponding MR images for one animal: lesions are numbered and a one to one correlation can be appreciated. Despite that different pulmonary lobes are shifted and flattened in the histologic slice and this is taken in the lung central region, with a risk of missing any lesions at the edge, a fairly good correlation (Pearson’s coefficient = 0.89) was obtained (Fig. 4B).

To verify the mechanism of action of PHA-848125, serial sections of the lungs were stained with two antibodies against CDK-related biomarkers, phosphoRb and cyclin A. A complete inhibition of both biomarkers expression was observed in the pulmonary malignant lesions of treated mice. A comparable inhibition was also observed in more differentiated tumors. In parallel, the proliferation rate, evaluated by Ki-67 staining, was strongly decreased in PHA-848125-treated samples (Fig. 5).

**Discussion**

Advances in the understanding of the molecular events underlying the development of lung cancer have revealed numerous potential new therapeutic strategies, including targeting EGFR, angiogenesis, and other signal transduction pathways. Some previous attempts to combine chemotherapy and targeted therapy in lung cancer...
have been unsuccessful, although the combination of the monoclonal antibody bevacizumab, which targets vascular endothelial growth factor, with chemotherapy, showed significantly longer survival times for patients with advanced nonsquamous NSCLC (30).

Preclinical testing of lung cancer therapeutics has been largely carried out using xenograft models. However, these models may not accurately mimic the behavior of lung tumors and poorly predict the clinical efficacy of anticancer agents.

Based on a growing understanding of the molecular alterations that most frequently occur in human lung tumors, several transgenic mouse models of NSCLC have been created (31). These models more accurately mimic the human disease and provide more predictive models in which to perform preclinical testing of new therapeutics.

K-ras is currently accepted to be the most frequently mutated oncogene in NSCLC. Emerging data suggest that K-ras mutations are negative predictors of benefit from both adjuvant chemotherapy and anti-EGFR–directed therapies (22, 32). Therefore, new therapies for patients with mutant K-Ras should be investigated in preclinical models of NSCLC with activated K-Ras.

The development of agents aimed to inhibit Ras-activated downstream pathways, such as the Cdk/Rb/E2F pathway, should be considered. At least three CDKs, CDK4, CDK6, and CDK2, and their regulators, control the progression from G1-S phase, whereas CDK1 is activated at the end of interphase and it is responsible for driving cells through mitosis. The present knowledge on the role of CDKs in controlling cell cycle progression indicates that a broad spectrum of activity versus different CDKs could be advantageous to bypass the potential compensatory mechanisms of cancer cells (33–35).

PH-A848125 is a pan CDK (CDK2, CDK1, and CDK4) inhibitor that showed a significant antitumor efficacy in various preclinical animal models (36). Here, we tested this compound in the K-RasG12D LA2 mouse model of NSCLC.

In parallel to the spread of new transgenic models, methodologic progress and advances in the instrumentation available for imaging small animals have provided an unprecedented opportunity to investigate these models noninvasively and test new therapeutic intervention.

To include the K-RasG12D LA2 model into a drug discovery program, we developed an imaging method to quantitatively measure total lung tumor burden. We performed a MRI study that involved a large cohort of mice and provided a detailed quantitative characterization of lung adenocarcinoma progression in a longitudinal study. We preferred an ECG and respiratory-triggered spin echo sequence instead of a faster gradient echo or echo planar imaging method because of the intrinsic minor sensitivity to susceptibility artifacts and the distinction of clearer boundaries of the lesions. We also found coronal sections more informative for tumor visualization and measurement: a relatively high in-plane resolution (156 μm) and thin slices (0.55 mm) allowed the detection of pulmonary nodules as small as 0.5 mm in diameter.

Validation of MRI data was necessary before using it extensively for measuring disease progression and therapeutic response. By enclosing in paraffin the whole organ, we have been able to evaluate the whole lung, but we induced a compression and a deformation of the organ; nevertheless, anatomic size relationships of the different pulmonary lobes were preserved and recognition of different lesions, observed three dimensionally on MR images, was feasible. In addition, a fairly good correlation (Pearson’s coefficient = 0.89) was obtained between lesion count by MRI and histology. Regions more prone to errors were those surrounding the heart and the big vessels, whereas tumors in the dorsal region were the most easily detected and accurately measured.
We evaluated the efficacy of our compound by two imaging methodologies, MRI and Cho-PET: a different set of animals was used for the two imaging experiments, which were not done in the same facility. Nevertheless, the same selection criteria by MRI were used for both imaging studies and immunohistochemistry evaluation.

PHA-848125 induced a significant tumor growth inhibition at the end of the treatment and this effect on the volume was also accompanied by a reduction in the cell membrane turnover, as seen by Cho-PET experiment. Although lung cancer is usually evaluated with 18F-FDG in the clinical practice, 11C-Choline was considered the tracer of choice. This was because FDG was highly up taken by the heart and could prevent a correct quantitation of the tracer uptake in the pulmonary masses. Although it is possible to reduce the heart uptake by fasting the mice, we opted for the choline tracer because it is poorly up taken by the myocardium and gives higher and more specific signal in correspondence of the lung masses; furthermore, it highlights both high- and low-grade tumors and this is advantageous in this mouse model.

MRI data were also analyzed according to the Response Evaluation Criteria in Solid Tumors criteria. Whereas the guidelines indicate the sum of longest diameters as a measure of dimension, here we used tumor volumes measured three dimensionally by covering the whole mass with thin adjacent slices. The general criteria of evaluation were otherwise preserved; as in the clinic, for example, we checked the absence of any new mass before assigning the state of stable disease; similarly, we chose to measure and consider only two or three lung lesions per animal and this is the case also in clinical studies, when evaluating organs with multiple lesions (27). We believe this data analysis adds value to this work by placing it in the context of a real preclinical trial and further helps the translation of these results to the clinic.

The mechanism of action of our compound, PHA-848125, whose efficacy in this model was proved by the two imaging modalities, was evaluated by immunohistochemistry. As expected, the proliferation index of lung lesions, by means of Ki-67, was found higher in adenocarcinoma areas than in adenoma regions and a strong decrease in its expression was observed in treated samples. In addition, a complete inhibition of both Rb phosphorylation, a direct CDK2 substrate, and Cyclin A expression was observed.

This study showed how multimodality imaging techniques can successfully be used to monitor tumor progression and response to treatment. Both MRI and PET methodologies allow a longitudinal monitoring of tumor development and response in a single animal; this allows for more clinically relevant study design and increases its statistical relevance. Imaging makes also possible to prescreen and select animals at a certain stage of the disease, as done in the present study as animal enrollment criteria, to reduce group variability and normalize the experiment.

Imaging studies, when coupled to standardized histologic methods, are highly valuable tools to support a broader application of genetically engineered mouse models in oncology discovery and development of new compounds.

PHA-848125, a promising new CDK inhibitor now in phase II clinical trial, showed significant efficacy in the K-RasG12DLA2 model of NSCLC and could represent a valid alternative to the current treatment of K-Ras–mutated forms of NSCLC. Interestingly, in a recent phase I study with this compound, a prolonged disease stabilization was observed in one patient with NSCLC (25).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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