Preclinical Optimization of MDM2 Antagonist Scheduling for Cancer Treatment by Using a Model-Based Approach

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Abstract

Purpose: Antitumor clinical activity has been demonstrated for the MDM2 antagonist RG7112, but patient tolerability for the necessary daily dosing was poor. Here, utilizing RG7388, a second-generation nutlin with superior selectivity and potency, we determine the feasibility of intermittent dosing to guide the selection of initial phase I scheduling regimens.

Experimental Design: A pharmacokinetic–pharmacodynamic (PKPD) model was developed on the basis of preclinical data to determine alternative dosing schedule requirements for optimal RG7388-induced antitumor activity. This PKPD model was used to investigate the pharmacokinetics of RG7388 linked to the time-course of the antitumor effect in an osteosarcoma xenograft model in mice. These data were used to prospectively predict intermittent and continuous dosing regimens, resulting in tumor stasis in the same model system.

Results: RG7388-induced apoptosis was delayed relative to drug exposure with continuous treatment not required. In initial efficacy testing, daily dosing at 30 mg/kg and twice a week dosing at 50 mg/kg of RG7388 were statistically equivalent in our tumor model. In addition, weekly dosing of 50 mg/kg was equivalent to 10 mg/kg given daily. The implementation of modeling and simulation on these data suggested several possible intermittent clinical dosing schedules. Further preclinical analyses confirmed these schedules as viable options.

Conclusion: Besides chronic administration, antitumor activity can be achieved with intermittent schedules of RG7388, as predicted through modeling and simulation. These alternative regimens may potentially ameliorate tolerability issues seen with chronic administration of RG7112, while providing clinical benefit. Thus, both weekly (qw) and daily for five days (5 d on/23 off, qd) schedules were selected for RG7388 clinical testing. Clin Cancer Res; 20(14); 3742–52. ©2014 AACR.

Introduction

The tumor suppressor p53 plays a key role in preventing malignant transformation and inhibiting the development of cancer. In response to cellular stress, the p53 pathway triggers gene transcription, which can result in cell-cycle arrest, apoptosis, and DNA repair and/or senescence (1). The dysregulation of the p53 pathway is the most frequent alteration detected in a broad range of human cancers, and approximately half of these cancers have been determined to carry mutated TP53 (2). Another mechanism by which p53 function may become lost is the overexpression of MDM2. Under nonstress conditions, the E3 ubiquitin ligase MDM2 is a negative regulator that directly binds to and inhibits p53 activity by targeting p53 for ubiquitin-dependent degradation (3, 4). Therefore, MDM2 overexpression results in reduced p53 levels, inadequate cell growth arrest, and apoptosis. Accordingly, disrupting the binding of MDM2 to a functional p53 can be expected to restore p53-dependent cellular arrest and apoptosis.

The nutlin family of MDM2 antagonists was designed to disrupt p53–MDM2 binding (3–7), thereby stabilizing and activating p53. In preclinical studies, the nutlin family member RG7112 promoted the reactivation of the p53 pathway to elicit growth arrest and apoptosis in tumor cells where MDM2 gene amplification or other oncogenic drivers have allowed evasion of p53-activated cell death (8). However, during RG7112 clinical testing, there were tolerability issues seen with chronic administration of RG7112, while providing clinical benefit. Thus, both weekly (qw) and daily for five days (5 d on/23 off, qd) schedules were selected for RG7388 clinical testing. Clin Cancer Res; 20(14); 3742–52. ©2014 AACR.

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challenges with prolonged daily oral administration of RG7112 (≥10 consecutive days in patients), including grade 3/4 vomiting, neutropenia, and thrombocytopenia (9). Therefore, optimization of the schedule of administration, including pursuing intermittent dosing, is an important consideration for further clinical development of MDM2 inhibitors.

The pyrrolidine RG7388, which has the same mechanism of action as the imidazoline RG7112, is a more potent and selective second-generation MDM2 inhibitor (8, 10). In preclinical studies, RG7388 effectively activated the p53 pathway in SJSA1 cells and induced tumor growth inhibition (TGI) in correlative xenografts in nude mice at significantly lower doses and exposures than RG7112 (10, 11). These results suggest the potential of achieving a clinical benefit at significantly lower drug concentrations with RG7388 and indicate that it should be possible to achieve a decrease in the severe toxicities that limited the less potent RG7112.

As RG7112 had poor tolerability when given daily, we sought to determine whether RG7388 could be dosed intermittently to circumvent the need for daily administration. To better understand the scheduling requirements for optimal RG7388 antitumor activity, a model-based approach was undertaken (12). First, a dynamic pharmacokinetic–pharmacodynamic (PKPD) model was developed to investigate how the pharmacokinetics of RG7388 are related to the time course of the antitumor effect in SJSA osteosarcoma xenografts in mice (Fig. 1, step 1 and 2). Second, the model was used to prospectively predict intermittent and continuous dosing regimens that would result in tumor stasis when tested in a follow-up efficacy study, predictions were in alignment with observed responses. These results supported the selection of the intermittent schedules utilized in the initial phase 1 RG7388 clinical trial to circumvent tolerability issues.

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**Translational Relevance**

Nutlins, small-molecule inhibitors of the p53–MDM2 interaction, reactivate p53 and have provided preclinical proof-of-concept as therapeutics for patients with tumors expressing functional p53. The nutlin family member RG7112 was the first small-molecule antagonist of MDM2 to be tested clinically. However, prolonged daily administration was poorly tolerated. Here, using the next-generation MDM2 antagonist RG7388, we demonstrate that intermittent regimens could be used instead of chronic administration, with the expectation of improving patient tolerability while inducing equivalent antitumor activity and efficacy. Modeling and simulation applied toward foundation data from an initial efficacy dataset facilitated a prospective evaluation of alternative regimens predicted to achieve tumor stasis. When tested in a follow-up efficacy study, predictions were in alignment with observed responses. These results supported the selection of the intermittent schedules utilized in the initial phase 1 RG7388 clinical trial to circumvent tolerability issues.
in tumor stasis (Fig. 1, step 3). This allowed for the testing of dosing hypotheses by comparing a priori predicted responses to the actual observed tumor growth profile (Fig. 1 step 4). Given that both p53 and MDM2 have short half-lives, two hypotheses were that continuous suppression of the p53–MDM2 interaction would be required for optimal antitumor activity, or alternatively, that stabilization and activation of p53 and subsequent apoptosis, trigger a sustained antitumor effect. The first hypothesis would be consistent with the need for continuous dosing and assumes that the PD is directly linked to the PK, whereas the second hypothesis implies that intermittent dosing should be sufficient to elicit a robust antitumor response, while potentially reducing the occurrence of severe adverse events associated with the prolonged continuous administration of RG7112 (Fig. 1, step 5).

Materials and Methods

In vitro testing in cancer cell lines

RG7388 was prepared at concentrations of 1 and 10 mmol/L in DMSO and stored in aliquots at −20°C. SJSA, RKO, HCT116, H460, A375, SW480, MDA435, and HeLa cells were obtained from the ATCC. Cell lines were authenticated by short tandem repeat analysis through Promega authentication services. For in vitro studies, cells were cultured in their ATCC-designated media. Medium was supplemented with 10% FBS and 1% 200 mmol/L l-glutamine. To assess cell viability, cells were seeded at densities identified for best growth for a 5-day assay in 96-well plates in normal growth media. Serial dilutions of RG7388 (1–3 in fresh media) starting at 300 μmol/L were applied to wells (1–10) in triplicate for a final concentration range of 0.01 to 30 μmol/L and control wells were treated with 0.3% DMSO equivalent to DMSO at the highest RG7388 concentration. Cell respiration, as an indicator of cell viability, was measured by the reduction of MTT to formazan as previously described (5).

Percent apoptosis was determined as described in Tovar and colleagues (8). For Western blot analysis, cells were cultured in T-75 flasks (4 mL total volume at 5 × 10⁶ cells/well) and incubated overnight at 37°C, 5% CO₂. Cells were treated with 0.3 or 1.8 μmol/L of RG7388 or 0.1% DMSO as control. Treatment duration was 16 hours, and lysates were prepared before washout and at 4, 8, 24, and 48 hours after RG7388 washout.

Western blot analysis

Antibodies against p53 and MDM2 were purchased from Santa Cruz Biotechnology. The anti-p21ΔN protein was purchased from Calbiochem, Merck KGAa and anti–β-actin from Sigma-Aldrich. Protein was extracted from cells or tumor tissue with 1× RIPA buffer (Sigma-Aldrich) containing protease inhibitors (Roche Diagnostics) by homogenization or scraping, respectively. Equal amounts of total protein were resolved on 4%–12% NuPAGE gradient gel (Invitrogen, Life Technologies) and blotted with antibodies as indicated. The chemiluminescent signal was generated with ECL Plus (GE Healthcare Life Sciences) and detected with a Fujifilm LAS-4000 imager. The densitometric quantitation of specific bands was determined using Multi Gauge Software (Fujifilm).

Pharmacokinetic analysis

To determine RG7388 plasma concentrations, blood samples were collected from female mice during in vivo antitumor studies. In each treatment group, on the first and/or last dosing day, blood samples (generally n = 2/time point) were collected at various predetermined time points ranging from 0.5 to 24 hours after the last dose. Pharmacokinetic assessment was performed via noncompartmental analysis using Watson v7.4 (Thermo Fisher Scientific), where parameters were calculated on the basis of the composite concentration–time data from each treatment group and sampling day. Sampling times were reported as nominal time, with concentrations below the limit of quantitation excluded. Parameters reported include plasma half-life (T½), Cmax, Tmax, and area under the plasma concentration–time curve from end of dosing to the last bleeding time point (AUC0–24 hours). The AUCs were calculated using the linear trapezoidal rule. The Cmax and Tmax values were taken directly from the plasma concentration–time profiles without extrapolations.

In vivo activity studies in xenograft tumor models

Athymic female nude mice (Crl:NU-Foxn1nu) were obtained from Charles River Laboratories. The health of all animals was monitored daily by gross observation and analyses of blood samples of sentinel animals. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee in our Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility.

At 10 to 12 weeks of age, mice were implanted with a 1:1 mixture of human SJSA osteosarcoma cells (ATCC) suspended in phenol-free Matrigel and PBS. Mice were implanted in the right flank at a concentration of 5 × 10⁶ cells in 0.2 mL total volume. At approximately day 10, animals were randomized according to tumor volume, so that all groups of 10 randomized mice had similar starting mean tumor volumes of 100 to 250 mm³.

Tumor measurements and weights were taken two to three times per week. TGI was calculated from percent change in mean tumor volume compared with the control group. Average percent weight change was used as a surrogate endpoint for tolerability in the experiment. Animals in each group were continuously followed beyond the last day of treatment to see whether tumor regrowth would occur. In this second phase of analysis, survival was calculated using a cutoff individual tumor volume of 1,500 mm³ as a surrogate for mortality. The increase in lifespan (ILS) was calculated as a percentage using the formula: [(median day of death in treated tumor-bearing mice) − (median day of death in control tumor-bearing mice)]/median day of death in control tumor-bearing mice × 100. Statistical analysis was performed as previously described (8).
RG7388 was administered as an amorphous solid dispersion microbulk precipitate powder containing 30% drug substance and 70% hydroxypropyl methylcellulose acetate succinate polymer that was reconstituted immediately before administration as a suspension in Kluce/Tween, and remaining suspension was discarded after dosing.

**Modeling and simulation**

The PKPD model structure. For PKPD modeling, plasma PK was fitted to a 1-compartmental model assuming nonlinear bioavailability and a first-order absorption and elimination rate constant. The differential equations and initial conditions for the PK are as follows:

\[
\frac{dA}{dt} = D \left( 1 - \frac{D^{\text{hill}}}{D^{\text{hill}} + D_0^{\text{hill}}} \right) - \tau A \quad A_0 = 0
\]

\[
\frac{dQ_c}{dt} = \tau A - \tau Q_c \quad Q_{c0} = 0
\]

where \(D\) corresponds to dose (mg/kg) and \(A\) represents unabsorbed drug. \(Q_c\) (\(\mu\)g) corresponds to the amount of drug in central compartment, \(\tau\) (hour-1) to the first-order absorption rate constant, \(ke\) (hour-1) to the first-order elimination rate constant, \(V\) (L/kg), and \(Cp\) (\(\mu\)g/mL) to, respectively, the volume and drug concentration in central compartment.

The full PKPD model assumes that the drug effects of RG7388 are delayed relative to the observed antitumor effect. This was described by using a signal distribution model based on the model proposed by Lobo and Balbhasar (13).

The model structure is shown in Supplementary Fig. S1, and the differential equations and initial conditions for the PKPD are as follows:

\[
\frac{dR}{dt} = k_g R - T_4 R, \quad R(0) = w_0
\]

\[
\frac{dT_1}{dt} = \tau a^{-1}(E - T_1), \quad T_{1(0)} = 0
\]

\[
\frac{dT_2}{dt} = \tau a^{-1}(T_1 - T_2), \quad T_{2(0)} = 0
\]

\[
\frac{dT_3}{dt} = \tau a^{-1}(T_2 - T_3), \quad T_{3(0)} = 0
\]

\[
\frac{dT_4}{dt} = \tau a^{-1}(T_3 - T_4), \quad T_{4(0)} = 0
\]

\[
E = k_2' C_p
\]

Where \(R\) represents the tumor volume, \(kg\) the exponential tumor growth rate, and \(w_0\) initial tumor volume (mm³), and \(tau\) (hour) represents the mean transit time equal for each compartment. Drug effect \(E\) was described by a linear drug effect model.

**PKPD parameter estimation.** PKPD parameter estimation was done using a nonlinear mixed effect modeling software Monolix, 4.3.1 (Lixoft). For the PK, residual errors were assumed to be proportional to the predicted concentrations and a proportional error model was selected. For the PD, an additional proportional error model was selected. Diagnostic plots were inspected to select the appropriate residual error model. The criteria for evaluating model performance was based on visual inspection, goodness of fit plots, including observed versus predicted plots, scatter plots of observed data overlay with predicted data, precision of the parameter estimates, and changes in the objective functions.

Simulations were conducted using software Berkeley Madonna, version 8.3.18, Copyright 1993-2001 Robert I. Macey & George F. Oster.

**IHC and image analysis**

Formalin-fixed, paraffin-embedded histologic samples from xenografts were used for IHC analysis using the Ventana Discovery XT platform (Ventana Medical System). Briefly, 5 micron tissue sections were stained using rabbit monoclonal antibodies anticleaved PARP-1 (Cell Signaling Technology) and anti-Ki67 (Thermo Fisher Scientific). Tissues were pretreated using heat-induced epitope retrieval, and a 3-step biotin-streptavidin-horseradish peroxidase detection method followed by diaminobenzidine (DAB) chromogen (Ventana Medical System) for antibody detection. Sections were counterstained with hematoxylin, dehydrated, and mounted with Permount mounting media. For image analysis, glass slides were scanned at x20 magnification using a Zeiss-Mirax digital slide scanner (Carl Zeiss Imaging), and Definis Tissue Studio software (Definis) was used to build custom-made image analysis algorithms. Generated algorithms were applied to the digital slides, to automatically detect and quantify viable xenograft areas, and areas of DAB deposition (positive antibody detection) within those viable regions. Numerical results were expressed as percentage of positively labeled area within the viable xenograft regions (percentage area of DAB labeling/xenograft viable area).

**Results**

**RG7388-induced apoptosis in SJS osteosarcoma cells is delayed relative to drug exposure but does not require continuous treatment**

Recent data demonstrated that RG7388 induces the p53 pathway activation and in vivo activity at much lower concentrations (~25%) and exposures (~7%-10%) than RG7112 (8, 10). Therefore, we initiated our experiments at these lower doses.

To investigate the dosing schedule requirements for RG7388-induced apoptosis of the MDM2-amplified SJS osteosarcoma line, cells were treated with a single dose of RG7388 (300 nmol/L or 1.8 μmol/L) for 16 hours as established in previous continuous-dose experiments.
These concentrations were chosen on the basis of antiproliferative activity as measured by an MTT assay and represent the average IC_{50} and six times the average IC_{50} for the P53 wild-type lines tested. Moreover, these concentrations are well below both the IC_{50} and IC_{90} levels observed for the 3 P53-mutant lines assayed, thus ensuring a sufficient concentration to elicit a functional p53-dependent response (10) while maintaining an insignificant likelihood of an off-target effect (Supplementary Table S1). At 24 and 48 hours after washout of RG7388, apoptosis was determined by positivity for annexin V. A significant apoptotic response over the control was induced with both the single 300 nmol/L and 1.8 μmol/L concentrations of RG7388 (P < 0.005), with the 1.8 μmol/L concentration providing optimal response (Fig. 2A). Induction of apoptosis was stronger at 48 hours post-washout compared with 24 hours post-washout. As comparable levels of apoptosis were achieved with 48 hours of continuous RG7388 dosing at the same concentrations (10), these in vitro results suggest that once the apoptotic component of the p53 pathway is activated in tumor cells by the MDM2 antagonist RG7388, continuous exposure to RG7388 is not required to sustain this activation.

Western blot analyses of p53, MDM2, and p21 protein levels (Fig. 2B) at the optimal apoptosis response level of 1.8 μmol/L further supported the idea that continuous dosing was not required for sustained RG7388 activity. P53 protein levels were increased 16 hours after a single treatment with RG7388. When p53 levels increase, the transcription of its targets should increase. Accordingly, the protein levels of MDM2 and p21 were also elevated. Although stabilized p53 protein did decrease 4 hours after washout, p53 levels then persisted and remained elevated compared with the control for at least 48 hours post-washout. In contrast, MDM2 and p21 protein levels were lost by 24 and 48 hours, respectively, post-washout. These patterns are consistent with previous observations made while treating cells continuously with RG7112 (8), and indicated that continuous dosing for RG7388 should not be required given its mechanism of action. Collectively these data along with efficacy data were utilized in the design of our modeling and simulation.

PKPD modeling to investigate the relationship between RG7388 exposure and time course of antitumor effect

The in vitro data suggest that the drug effect is delayed and then maintained relative to the drug exposure even after washout. To further understand how the pharmacokinetic of RG3788 is linked to the antitumor effect, we conducted a PKPD study in SJSA osteosarcoma xenograft-bearing mice. In this study, the tumor growth profiles and the respective plasma PK profiles were monitored over a 21-day period for inclusion in the model.

Animals were given oral doses of RG7388 at 1.11, 3.33, 10, or 30 mg/kg once daily, 50 mg/kg once weekly, or 50 mg/kg twice weekly (Monday/Wednesday). The highest doses investigated were of predetermined tolerability based on pilot experiments (data not shown). Although the once-daily dose of 30 mg/kg of RG7388 (total 210 mg/kg/wk) seemed to be the most effective dose at inhibiting tumor growth (>100% TGI with 9 partial regressions), the 50 mg/kg of RG7388 given twice a week (total 100 mg/kg/wk) was equivalent by statistical analysis (Fig. 2C; 96% TGI with 3 partial regressions; P > 0.05). In addition, 50 mg/kg of RG7388 given once a week (50 mg/kg/wk) elicited an
equivalent TGI as 10 mg/kg of RG7388 given once daily (70 mg/kg/wk; 79% TGI vs. 77% TGI, respectively; $P > 0.05$), despite being a lower total weekly dose. Therefore, intermittent dosing was equivalent to continuous administration for RG7388-mediated TGI in this xenograft model.

RG7388 exposure levels after multiple oral doses in female mice
RG7388 exposure levels were determined in the mice after the last dose of RG7388 (i.e., day 21). The exposures increased greater than dose proportionally between 1.11 mg/kg and 3.33 mg/kg, and less than dose proportionally between 3.33 mg/kg and 50 mg/kg (Fig. 3A and Supplementary Fig. S2). The concentration–time profiles were well described by a one-compartmental PK model (Supplementary Figs. S2 and S3).

The dose–exposure response relationship was characterized quantitatively and a mechanism-based PKPD model was fitted simultaneously to the observed individual PK and PD data of all groups (Fig. 3A; PKPD parameter estimates Supplementary Table S2). The model separates

Figure 3. Predicted and observed tumor growth data. A, observed and model-predicted tumor growth profiles. Modeling and simulation of PK/PD from initial experiment was applied to predict intermittent doses/regimens that would elicit tumor stasis. B, intermittent dosing regimens expected to result in 100% TGI.
system-related properties (tumor size and tumor growth rate) from drug-related properties (potency, drug-induced kill rate) and incorporates the following assumptions: (i) MDM2 is amplified, resulting in the inhibition of p53-induced apoptosis in the SISA xenograft tumors, (ii) proliferation of SISA cells thereby becomes the predominant process, resulting in unperturbed tumor growth, (iii) after administration of RG7388, MDM2 is sufficiently blocked from negatively regulating p53, which in turn induces cellular growth arrest and apoptosis, and (iv) the delay in tumor response to drug effect is due to signal transduction processes intervening between drug–receptor interaction and the killing event with the killing effect occurring through a signal transduction cascade (14). Similar modeling approaches have been reported to assess antitumor effect in xenograft mice (15, 16). These models allow for the estimation of a concentration resulting in tumor stasis. The drug effect of RG7388 is assumed to depend on its plasma concentration \([C(t)]\) and the tumor volume. The tumor volume predictions for different doses of RG7388 versus experimental data are shown (Fig. 3A).

Model-based approach to select intermittent dosing regimens targeting tumor stasis

This model was then used to select intermittent dosing regimens that are predicted to result in tumor stasis. The PKPD model quantitatively characterized how the pharmacokinetics of RG7388 is linked to the antitumor effect in SISA osteosarcoma. This in turn enabled prospective prediction of intermittent and continuous dosing schedules targeting tumor stasis (Fig. 3B). A variety of dosing regimens were selected on the basis of the initial clinical plan of a 28-day schedule, ranging from daily dosing up to seven times weekly, to five daily doses followed by a 23-day drug holiday.

All of the intermittent dosing schedules suggested by modeling and simulation (M&S) were tested experimentally and confirmed profound reduction in mean tumor volume compared with vehicle control (\(P < 0.05\) all vs. control; Fig. 4). In addition, survival was also examined, and all of the dosing schedules resulted in increased life span compared with vehicle control (\(P < 0.05\) all vs. control; Fig. 4). Several of the intermittent dosing regimens demonstrating dramatic TGI and increased lifespan were statistically equivalent (\(P > 0.05\)). These regimens included an 80 mg/kg dose given on a 5 days-on and 23 days-off schedule (>100% TGI with 9 partial regressions and a 127% ILS), 200 mg/kg (two 100 mg/kg doses administered 8 hours apart) given once a week (>100% TGI with 7 partial regressions and a 162% ILS), and chronic administration at 30 mg/kg given daily for 28 days (>100% TGI with 7 partial regressions and a 127% ILS). The tumor volume changes predicted by M&S were in alignment with the generated data, demonstrating the robustness of the modeling techniques.

Induction of apoptosis and antiproliferation in vivo with single dose and short schedule (5-day) RG7388 administration

The pharmacodynamic effects of the highest RG7388 dose based on traditional tolerability measures (i.e., weight loss) and predicted for intermittent dosing (5 days of 80 mg/kg) by the M&S was then compared with both the highest feasible single dose of 200 mg/kg (based on viscosity limitations of the suspension) and the 80 mg/kg dose in SISA xenograft tumors. Effects on apoptosis were examined by BLI of activated caspases-3 and -7. Activated caspases-3 and -7 are considered to be an early sign of apoptosis, as their presence can be detected before plasma membrane blebbing and DNA fragmentation. Bioluminescent detection of activated caspases-3 and -7 was monitored in SISA1-luc2 tumor-bearing mice using Z-DEVD-aminoluciferin as a substrate for activated caspases-3 and -7. When the DEVD peptide sequence is cleaved by the activated caspases, aminoluciferin is released and becomes a substrate for the luciferase enzyme produced by SISA-luc2 cells. Therefore, the luminescent signal is emitted only in apoptotic cells. An additional marker of apoptosis, PARP, was examined. PARP is a substrate of caspase-3 and is cleaved to cPARP-1 during
apoptosis. cPARP-1 was detected using IHC. Antiproliferation was also examined via IHC using Ki67, which is a cellular marker specific for proliferating cells.

At 48 hours after a single dose of RG7388, tumors from treated mice produced a maximal statistically significant induction in luciferase signal (Fig. 5A), indicative of apoptosis via activated caspases-3 and -7, at the 80 mg/kg and 200 mg/kg dose levels as compared with vehicle controls ($P < 0.05$). Representative bioluminescent images from mice bearing vehicle and RG7388-treated tumors show the dose-dependent increase in luminescence and, therefore, apoptosis (Fig. 5A). The apoptotic response to RG7388 was also examined by measuring cPARP-1 via IHC. Similar to activated caspase-3 and -7, the largest increase in cPARP-1 levels versus vehicle control was seen at 48 hours after a single dose of 200 mg/kg ($P < 0.05$; Fig. 5B). In addition, IHC examination of the proliferation marker Ki67 revealed the most significant decrease in positive staining at 48 hours after a single 200 mg/kg dose compared with vehicle control ($P < 0.05$; Fig. 5C).

When RG7388 was administered for 5 days at 80 mg/kg, the maximal effect on apoptosis via activated caspase-3 and -7 levels versus vehicle control was observed on day 3 ($P < 0.05$; Fig. 6A). Moreover, cPARP-1 levels were highest on day 3 versus vehicle control after 5 days of RG7388 administration (Fig. 6B). Finally, with 5 days of administration of RG7388, the maximal decrease in Ki67 versus vehicle control also occurred on day 3 ($P < 0.05$; Fig. 6C). Therefore, apoptotic and antiproliferative pharmacodynamic effects were comparable with the continuous (80 mg/kg daily) and with the single dose (200 mg/kg) dosing regimen. This supports our model-based hypothesis that a continuous dosing regimen is not needed.
Discussion

MDM2 inhibitors can be used in MDM2 gene amplified tumors to restore functional p53 activity. In preclinical models, RG7388, a second-generation MDM2 inhibitor, has demonstrated the ability to activate apoptosis and induce tumor stasis with continuous treatment (10). Here, we provide evidence that continuous dosing of RG7388 is not required for sustained activity. Although in vitro apoptotic effects were delayed relative to noncontinuous treatment, once triggered, the p53 pathway activation of apoptosis was irreversible in SISA1 cells even after drug removal. Interestingly, Western blot analysis showed that p53 levels remained elevated significantly longer than MDM2 and p21 after RG7388 washout. The loss of MDM2 and p21 protein levels could be the result of changes in protein half-life on p53 activation as evidenced in published results where accelerated MDM2 autodegradation was reported during p53-mediated DNA damage response (17). However, p53 function and stability may also be tightly controlled by its subcellular localization (18, 19). Therefore, it is possible that in a cell triggered into apoptosis, nuclear or mitochondrial localized p53 remains fairly stable following RG7388 washout, whereas MDM2 and p21 are accessible to be targeted by the cytoplasmic ubiquitin proteasome pathway. From a molecular standpoint, the continued presence of p53 after RG7388 removal may be critical for sustained activity. These observations were also made with RG7112 but necessitated much higher drug concentrations than the more potent RG7388 to retain elevated p53 levels on washout (data not shown). Collectively, our in vitro results support the hypothesis that RG7388 can induce sustained antitumor activity when given on an intermittent dosing schedule. However, as previous studies in cell lines have shown differential apoptotic response (6, 8), we must concede that additional investigation is warranted in other MDM2 antagonist sensitive xenograft models. Furthermore, p53 persistence as seen in the SISA1 after washout must be confirmed in other cell lines where significant differences in growth characteristics and phenotypes will perhaps necessitate optimized-type specific dosing schedules. For the studies outlined in this report, the SISA1 represents an ideal proof-of-concept model to investigate the feasibility of this approach.

An intermittent dosing schedule is expected to reduce the potential for and/or severity of adverse events in clinical trials such as hematologic toxicities because it allows the system to recover. Drug-induced thrombocytopenia and neutropenia observed with RG7112 is assumed to act on progenitor cells in bone marrow. Therefore, a decrease of platelets and/or neutrophils in circulation occurs with a lag time relative to drug treatment. Washout of the drug is then followed by a rebound and return to baseline of the blood cells after replenishment and completed maturation phase of the progenitor cells.

A model-based approach was thereby applied to guide the selection of intermittent dosing regimen achieving tumor stasis. As a first step, a PKPD study was conducted to relate the pharmacokinetics to the time course of the anticancer effects by modeling. A mechanism-based PKPD model was developed assuming a delayed response relative to the drug concentration motivated by the in vitro findings showing a delayed apoptotic response relative to RG7388 washout (data not shown). Collectively, our in vitro results support the hypothesis that RG7388 can induce sustained antitumor activity when given on an intermittent dosing schedule. However, as previous studies in cell lines have shown differential apoptotic response (6, 8), we must concede that additional investigation is warranted in other MDM2 antagonist sensitive xenograft models. Furthermore, p53 persistence as seen in the SJSA1 after washout must be confirmed in other cell lines where significant differences in growth characteristics and phenotypes will perhaps necessitate optimized-type specific dosing schedules. For the studies outlined in this report, the SJSA1 represents an ideal proof-of-concept model to investigate the feasibility of this approach.

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exposure. Similar modeling approaches have been reported to assess antitumor effect in xenograft mice (15, 16). These models allow for the estimation of a concentration resulting in tumor stasis. Rocchetti and colleagues (20) demonstrated that this estimated concentration correlated with typical therapeutic doses for select chemotherapeutic agents, emphasizing the clinical translational potential of this approach. The tumor stasis concentration can be regarded as a reference concentration to be achieved in vivo for attaining significant anticancer activity. In addition, Luo and colleagues (21) demonstrated, for cetuximab, that the active serum concentration determined in a preclinical xenograft model (average concentration at steady state) correlated well with the active drug concentration achieved in patients with cancer. As with any model, there are associated limitations. Our model assumes the tumor mass to consist solely of tumor cells and does not consider the interaction between stroma and tumor compartment. Moreover, drug-induced resistance, which can certainly influence outcome, is excluded. Nonetheless, our semimechanistic model described the PK and time course of tumor growth across all doses reasonably well. Therefore, based on these modeling results, simulations were conducted to identify intermittent dosing regimens achieving tumor stasis. A follow-up study was conducted to evaluate the observed tumor growth curves compared with the prospectively predicted profiles.

Both daily and intermittent dosing with RG7388 was effective at achieving tumor stasis as predicted by the PKPD model. Therefore, despite the inherent short half-lives of p53 and MDM2, continuous suppression of the p53–MDM2 interaction was not required for optimal antitumor activity. Pharmacodynamic effects of single versus short-term (5 days) dosing were examined in vivo using the MDM2-amplified SJSA1 osteosarcoma model. In vivo analysis of apoptosis and antiproliferative effects revealed that the single highest feasible dose of RG7388 (200 mg/kg) was sufficient to activate apoptosis and decrease proliferation, with a maximal effect at 48 hours. Likewise, a dose predicted as optimal via M&S on a 5-day schedule (80 mg/kg) also activated apoptosis and decreased proliferation, with a maximal effect after 3 days.

RG7112, a cis-imidazoline, and RG7388, a pyrrolidine compound, both bind to MDM2 and prevent the association of MDM2 with p53. Although these compounds have a shared mechanism of action and both reversibly bind to MDM2, RG7388 can be used at significantly lower doses due to superior potency and specificity (8, 10). These superior properties of RG7388 may also allow RG7388 to be dosed intermittently, which should circumvent toxicities associated with prolonged continuous treatment with RG7112 while maintaining clinical benefit. The preclinical data presented herein provide proof-of-principle that intermittent RG3788 dosing can provide the same activity as daily dosing.

Although RECIST responses were achieved in previous RG7112 clinical trials, patients had difficulty tolerating RG7112 given on the requisite daily schedule (9). On the basis of those results and on the work presented here, a once weekly × 3 followed by 13 days of rest, 28-day cycle schedule, and a 5 consecutive days of a 28-day cycle schedule were utilized for the initial phase I RG3788 clinical trial (trial registration identifier: NCT01462175). The ability to administer RG7388 intermittently should provide better tolerability for patients, which may in turn result in more robust clinical responses.

Disclosure of Potential Conflicts of Interest

B. Higgins is an employee of Roche. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Higgins, G. Kelli, A. Walz, Z. Filipovic, E. Lee, K. Kolinsky, S. Tannu, R. Garrido, M. Linn, K. Packman
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Higgins, A. Walz, C. Tovar, Z. Filipovic, K. Kolinsky, S. Tannu, C. Meille, K. Packman

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