Adjuvant Sirolimus Does Not Improve Outcome in Pet Dogs Receiving Standard-of-Care Therapy for Appendicular Osteosarcoma: A Prospective, Randomized Trial of 324 Dogs

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ABSTRACT

Purpose: The mTOR pathway has been identified as a key nutrient signaling hub that participates in metastatic progression of high-grade osteosarcoma. Inhibition of mTOR signaling is biologically achievable with sirolimus, and might slow the outgrowth of distant metastases. In this study, pet dogs with appendicular osteosarcoma were leveraged as high-value biologic models for pediatric osteosarcoma, to assess mTOR inhibition as a therapeutic strategy for attenuating metastatic disease progression.

Patients and Methods: A total of 324 pet dogs diagnosed with treatment-naïve appendicular osteosarcoma were randomized into a two-arm, multicenter, parallel superiority trial whereby dogs received amputation of the affected limb, followed by adjuvant carboplatin chemotherapy ± oral sirolimus therapy. The primary outcome measure was disease-free interval (DFI), as assessed by serial physical and radiologic detection of emergent macroscopic metastases; secondary outcomes included overall 1- and 2-year survival rates, and sirolimus pharmacokinetic variables and their correlative relationship to adverse events and clinical outcomes.

Results: There was no significant difference in the median DFI or overall survival between the two arms of this trial; the median DFI and survival for standard-of-care (SOC; defined as amputation and carboplatin therapy) dogs was 180 days [95% confidence interval (CI), 144–237] and 282 days (95% CI, 224–383) and for SOC + sirolimus dogs, it was 204 days (95% CI, 157–217) and 280 days (95% CI, 252–332), respectively.

Conclusions: In a population of pet dogs nongenomically segmented for predicted mTOR inhibition response, sequentially administered adjuvant sirolimus, although well tolerated when added to a backbone of therapy, did not extend DFI or survival in dogs with appendicular osteosarcoma.

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Translational Relevance

mTOR is a serine/threonine kinase that serves as a key nutrient sensor that regulates diverse cellular functions, including ribosome biosynthesis, protein translation, growth, and cytoskeletal rearrangement. Identified as a driver of metastasis, mTOR and its downstream signaling pathways mediated by mTORC1 and mTORC2, serve as attractive druggable targets for delaying metastatic progression in solid tumors, including osteosarcoma. However, given osteosarcoma's orphan disease status, the scalable and rapid assessment of mTOR inhibitors used in the upfront adjuvant setting would be protracted, necessitating the employment of higher throughput model systems that faithfully recapitulate the biologic complexities of the human disease. This study utilizes canine patients with osteosarcoma as a means to efficiently evaluate the mTOR inhibitor, sirolimus, as a potential antimetastatic agent. Our results provide high-value biologic evidence for the translational value of canine osteosarcoma for prioritizing novel antimetastatic strategies that might be advanced for pediatric osteosarcoma clinical trials.

Introduction

Osteosarcoma is a common and aggressive spontaneous malignancy arising from osteoblast lineage and affecting two primary species, human beings and canines. Comparatively, osteosarcomas in both people and dogs share conserved clinical, molecular, genetic, and biological behaviors (1–6). Despite significant efforts to identify and implement treatment strategies that provide durable tumor control, metastatic osteosarcoma progression continues to be a leading cause of death for both human and canine patients. For humans, significant improvements in outcome have not occurred in more than 30 years since the implementation of multiagent chemotherapy alongside limb-salvaging surgical procedures, with approximately 30% of patients developing metastatic disease despite aggressive first-line treatment (7, 8). Similarly, clinical data collected from pet dogs with naturally occurring osteosarcoma enrolled on both retrospective and prospective trials consistently demonstrate survival times that range from 242 to 306 days, with uniformly prescribed treatment being amputation of the affected limb, followed by adjuvant cytotoxic chemotherapy (9). Collectively, there are resounding scientific and clinical justifications for exploring complementary and orthogonal modeling paradigms that might efficiently and rapidly validate molecularly targeted agents for curbing metastatic osteosarcoma progression (10).

The Osteosarcoma Project is a joint initiative launched by the Morris Animal Foundation, the QuadW Foundation, and the NCI's Comparative Oncology Trials Consortium (NCI-COTC) to identify new therapeutic interventions that prevent or delay metastatic progression in osteosarcoma via screening of novel agents in pet dogs with spontaneously arising disease. The initiative is designed to compare investigational agents against a prospectively enrolled cohort of dogs receiving the current standard of care (SOC) for osteosarcoma, which is limb amputation, followed by four doses of carboplatin chemotherapy. Through this collaborative partnership, the conductance of these rapid and scalable clinical trials in pet dogs is expected to provide unparalleled translational insights and discoveries related to osteosarcoma metastatic progression, which might be leveraged by consortiums, such as the Children's Oncology Group or Sarcoma Alliance for Research through Collaboration, for clinical guidance and target prioritization in pediatric patients with osteosarcoma.

Using metrics for valuing preclinical data types for prioritizing and advancing agents to be assessed in pediatric osteosarcoma trials (11), a group of clinician-scientists endorsed sirolimus as the first agent to be evaluated within the Osteosarcoma Project clinical trial framework. Robust scientific, preclinical, and translational justification for the selection of sirolimus as a favorable agent with presumed antimetastatic activities are multifactorial. First, the PI3K/mTOR pathway has been identified as a central signaling pathway responsible for mediating multiple aspects of osteosarcoma progression and metastases (12, 13). Second, inhibition of the mTOR pathway using sirolimus has been assessed in orthotopic mouse models of metastatic osteosarcoma (14, 15), and at clinically achievable exposures, sirolimus exerts robust antimetastatic activities that are distinct from a modest effect on heterotopic primary tumor growth in mice (16). Third, clinical data collected within a series of canine comparative oncology trials carried out in normal and tumor-bearing dogs demonstrate that sirolimus administered parenterally is tolerable and provides pharmacokinetic exposures that are translatable to those achieved in human patients, and results in effective tumoral and surrogate peripheral blood mononuclear cell (PBMC) modulation of pS6RP, a proximate target of the mTOR pathway (17, 18). Finally, an intriguing study performed by the French Sarcoma Group reported the off-label use of sirolimus alone or in combination with cyclophosphamide for the management of refractory relapsed osteosarcoma, and demonstrated that sirolimus could produce disease stabilization in a subset of patients with advanced metastatic osteosarcoma (19).

These existent data generated in preclinical models (mice/dogs) and clinical findings in human patients with advanced-stage disease, in conjunction with provocative role of mTOR signaling in osteosarcoma cellular biology related to invasion, proliferation, survival, and metastasis (15, 20–22), served as the impetus for conducting the clinical trial reported herein. The study hypothesis was that adjuvant sirolimus therapy administered sequentially following SOC therapy will exert antimetastatic activities and extend the disease-free interval (DFI) for dogs receiving it compared with those receiving only SOC by

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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at least 50%. The primary endpoints of the trial included DFI and overall survival assessed in a prospective, randomized clinical trial setting. A secondary objective of this study was to identify key factors related to tolerability and clinical efficacy of sirolimus when studied in the minimal residual disease setting, including pharmacokinetic parameters and patient/tumor-related factors.

Patients and Methods

Trial design

The NCI-COTC

The COTC infrastructure provides a facile means of conducting multicenter clinical trials in pet dogs to advance anticancer drug development and cancer biology questions that are not sufficiently asked or answered in other animal models (23, 24). Eighteen COTC member institutions participated in this randomized, two-arm, parallel superiority trial following CONSORT guidelines. The study period included the entirety of the dogs' DFI after diagnosis, amputation of the affected limb, and administration of adjuvant carboplatin therapy with or without adjuvant sirolimus administration. Dogs were considered off-study at the time metastatic disease progression was detected and confirmed through standard clinical radiographic imaging methods and/or tissue analysis.

Participants

Patient enrollment procedures and eligibility criteria

Dogs met predetermined eligibility criteria (Supplementary Table S1) to participate in the trial. Each participating COTC member institution obtained and maintained approval from their respective Institutional Animal Care and Use Committee prior to enrolling patients in this trial. Dogs were actively recruited into the clinical trial over a span of 31 consecutive months, with patient-specific finalized outcomes reported up to 3 years post-enrollment.

Sample size calculation

A sample size calculation was performed to estimate the number of dogs needed to detect a difference in DFI of 6 months [SOC, 282 days and SOC + sirolimus (SOC + S), 464 days] using a two-sided log-rank test at 80% power and at a significance level of 0.05 [PASS 13 Power Analysis and Sample Size Software (2014), NCSS, LLC, ncss.com/software/pass].

Randomization and allocation

Prior to limb amputation, dogs were randomized to either SOC or SOC + S arms in an allocation ratio of 1:1. Arms were stratified in a 2 × 2 matrix with regards to two consistent known prognostic factors (25), being tumor location (proximal humerus vs. nonproximal humerus) and alkaline phosphatase (ALP) status (normal vs. elevated), by assigning dogs to one of the four blocks. Dogs were randomized using a pregenerated block randomization list (with four dogs in each block), through generation of random number sequences for each block [using RAND() function] in commercially available software (Microsoft Excel for Mac version 16.16, Microsoft). Randomization to SOC or SOC + S was instituted at the initiation of carboplatin chemotherapy instead of prior to surgery, and treatment allocation was not blinded to enrolling COTC investigators or dog owners.

Clinical procedures

Biologic sample collections and biobanking efforts

This study protocol included prospective collection of biologic samples (**Fig. 1A** and **B**; Supplementary Fig. S1) to enable *post-hoc* analyses of factors relating to metastatic behavior of the primary tumor. Each dog had whole blood, PBMCs, serum, tumor, and normal tissue collected at time of surgery, prior to initiation of any therapy. In addition, dogs enrolled on the SOC + S arm had whole blood, PBMCs, and serum collected during five pharmacokinetic sampling curves over seven sampling timepoints across the four cycles of sirolimus exposure.

Surgery

Dogs underwent either forelimb or hindlimb amputation surgery with regional lymphadenectomy to allow baseline biologic sample collection and tissue banking. At the time of surgery, tumor and normal tissue samples, serum, whole blood, and PBMCs were collected and stored for future analysis. Surgery occurred within 10 days of study enrollment.

Carboplatin chemotherapy

Between 10 and 21 days postamputation, dogs began carboplatin chemotherapy at a dosage of 300 mg/m^2 i.v. If dogs had unacceptable clinical laboratory findings to allow for safe chemotherapy administration (e.g., grade 1 or higher hematologic toxicity), the COTC clinician prescribed a dose delay of ≤7 days. In dogs with a history of a treatment delay due to grade 2 or higher myelosuppression, a 10% reduction in carboplatin was prescribed for the ensuing cycle, but the every 21-day treatment interval was preserved as often as possible.

Sirolimus administration

Sirolimus walk-in trial: A dose-confirming study was conducted in tumor-bearing dogs prior to initiation of the randomized trial of SOC ± S to determine the tolerability, pharmacokinetics, and optimized dosing regimen of oral sirolimus. A total of 22 tumor-bearing dogs received sirolimus orally at 0.1 mg/kg on either a Monday through Friday (M–F) schedule or Monday-Wednesday-Friday (M/W/F) schedule for 4 consecutive weeks. Whole-blood samples were collected from a subset of dogs over a 48-hour period (0, 1, 2, 4, 6, 8, 24, and 48 hours) following the first and last dose of sirolimus (administered on days 1 and 26), as well as single-timepoint measurements on days 8 and 19, to monitor drug levels during treatment. Tolerability of both dosing schemes was assessed in all 22 dogs, while pharmacokinetic parameters were assessed in a subset of four dogs in the M–F dosing schedule, and three dogs in the M/W/F dosing schedule. Sirolimus tablets (0.5 and 2 mg) formulated for human use were used for treatment of pet dogs within both the walk-in and SOC + S trials (sirolimus generic tablets for oral use, 0.5 and 2 mg, Greenstone Brand, Greenstone LLC).

Adjuvant SOC + S arm: For dogs randomized to the SOC + S arm, treatment with sirolimus began within 7 days after completion of fourth cycle of carboplatin dosing at the week 15 visit if dogs were confirmed free of macroscopic metastatic disease through the conductance of physical examination and thoracic radiography. Sirolimus was administered on a 4 days on/3 days off (treatment Monday–Thursday, with no treatment Friday–Sunday) regime at a dose of 0.1 mg/kg orally once a day for a 26-day cycle. The planned treatment interval was four consecutive cycles of sirolimus treatment. A seven-point whole-blood pharmacokinetic sampling curve was collected on days 11 and 25 of cycle 1 of sirolimus treatment; for cycles 2–4, only a day 25 curve was conducted. Each dog had three-view thoracic radiographs completed at the end of cycles 2 and 4, with evaluation by a board-certified veterinary radiologist.

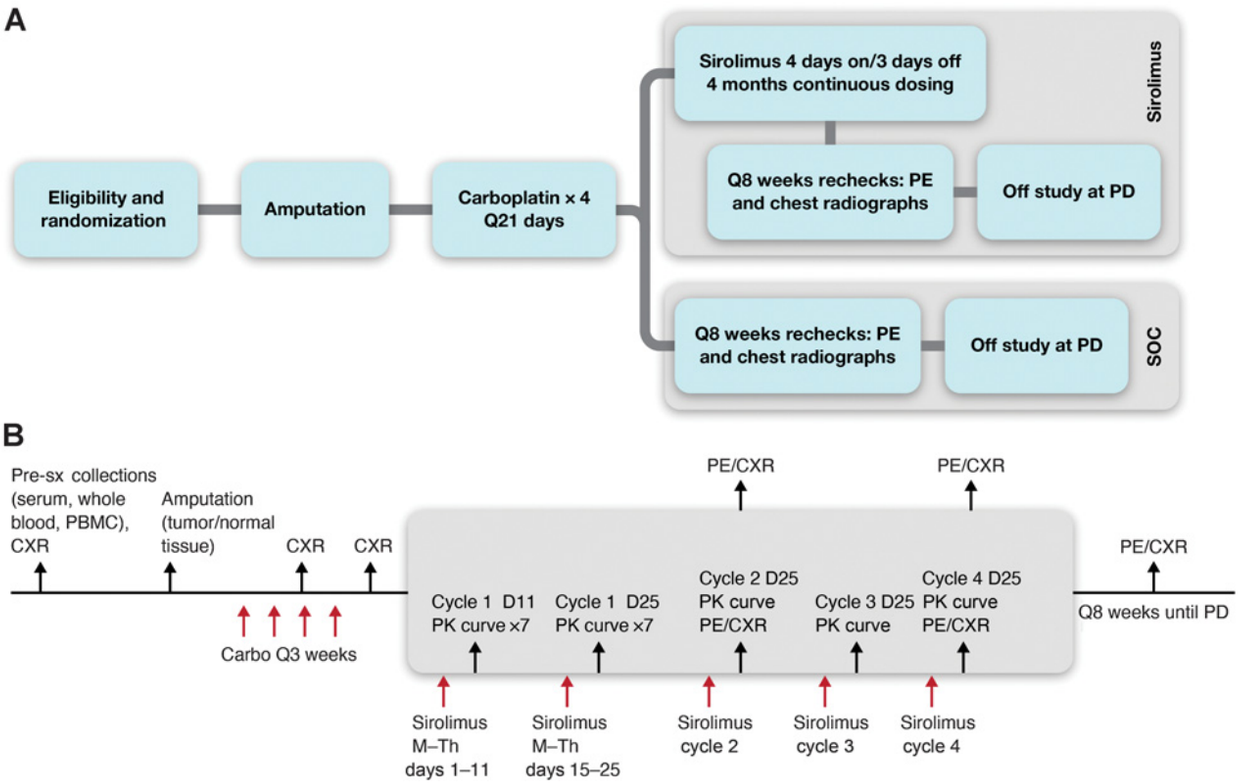

Figure 1.

Overview of schema (**A**) and schedule of study procedures (**B**) for canine patients with osteosarcoma enrolled in the SOC vs. SOC + sirolimus (SOC + S) clinical trials. CXR, three-view thoracic (chest) radiographic assessment; PD, progressive disease; PE, physical exam; PK, pharmacokinetic assessment of drug levels; Sx, surgery; Q3 week, every 3 weeks; Q21 day, every 21 days.

Pharmacokinetic assessment of sirolimus-treated dogs

A validated method for extraction of a sirolimus analogue was used as described to extract sirolimus from 100 μL of each blood sample by means of protein precipitation with 0.2 mol/L zinc sulfate, followed by liquid–liquid extraction with 1 mL of ethyl acetate (26). The sirolimus concentration in each sample was determined by use of LC/MS-MS with tacrolimus as an internal standard as described previously (18).

Initial pharmacokinetic analysis involved full time-course samples collected on days 11 and 25 of cycle 1 for 20 random dogs. These full sample sets were used for the development of a limited sampling approach. This was done to determine the necessity of analyzing full time-course samples for the estimation of drug exposure via AUC and trough drug levels. Multiple stepwise linear regression modeling was done using six random subsets of the complete dataset and a consensus model was developed utilizing the 2- and 8-hour timepoints for estimation of AUC_{0-24h} (MATLAB vR2019a, MathWorks).

$$\mathrm{AUC(ng/mL \times hour)} = 3.83 + (C_{2h} \times 3.15) + (C_{8h} \times 16.72).$$

The predictive capability of the limited sampling model was determined by calculation of the median absolute performance error (MAPE%), the median performance error (MPE%), and the root mean squared performance error (RMSPE%), as described previously (27). These analyses showed the MPE% = 1.89, MAPE% = 3.41, and RMSPE% = 6.37 and an accuracy ± precision (%CV) of the prediction as 95.3% ± 4.4%.

Clinical monitoring

Clinical monitoring was carried out through physical examination and thoracic radiography according to a standardized schedule (**Fig. 1A** and **B**; Supplementary Fig. S1). When clinically indicated, additional diagnostics to confirm or deny the presence of metastatic disease or other comorbidities were performed. Acute or chronic toxicities attributable to study procedures, surgery or drug treatments, or disease progression were prospectively assessed within this trial design. Adverse events (AE) were given an attribution based upon a group consensus discussion between the COTC investigator, the study principal investigators (T.M. Fan and A.K. LeBlanc), and the NCI COP study coordinator (C.N. Mazcko). After completion of chemotherapy and/or sirolimus, dogs were reevaluated every 8 weeks with a physical examination and three-view thoracic radiographs. If progressive pulmonary metastatic disease was suspected, repeat thoracic radiographs were performed after an additional 3–4 weeks to confirm the observation. All dogs were followed with this clinical monitoring schedule until confirmation of progressive disease, and/or until 3 years had passed from the date of surgery, whichever came first.

Statistical analysis

Categorical variables were described using frequencies and percentages. Continuous variables were assessed for normality using skewness, kurtosis, and Shapiro–Wilk tests. Baseline characteristics were compared between SOC and SOC + S groups (age, weight, gender,

tumor location, and ALP status) using χ^2 or Fisher exact test for categorical variables and Kruskal–Wallis tests for continuous variables.

Kaplan–Meier methods were used to generate survival curves for SOC and SOC + S groups and to calculate median DFI and median overall survival time with 95% confidence intervals (CI). The DFI was calculated as the number of days from the date of the limb amputation to the date of first detection of metastases. Dogs were censored in the DFI analysis if they did not have metastases documented at the time of last follow-up if alive and on study, or at death, or the date the dog went off study. The overall survival time was calculated as the number of days from limb amputation to death due to disease as gleaned from either clinicians' observations or necropsy results (for disease-specific survival). Dogs were censored in the survival analysis if they were alive at last follow-up or were lost to follow-up. Log-rank tests were used to evaluate for difference in DFI and overall survival time between SOC and SOC + S groups. Log-rank tests were also used to assess for associations between baseline risk factors, including serum ALP and anatomic tumor site, within SOC and SOC + S groups. A subgroup analysis of dogs in the SOC + S group was performed using Cox proportional hazards analysis to assess for associations between sirolimus variables (mean AUC baseline and grade 3 or above AEs), variables on DFI, overall survival time, and survival time after development of metastasis. Statistical significance was set at $\alpha = 0.05$ and the statistical analysis was performed using commercially available software (SAS software, version 9.4 of the SAS System for PC. Copyright © 2013 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc. and GraphPad Prism for Windows, version 7).

An intent-to-treat and per-protocol analysis were performed and reported. The modified intent-to-treat analysis included all dogs randomized to each treatment group and prescribed the chemotherapy protocol. The per-protocol analysis included dogs that completed the prescribed chemotherapy protocol and were considered evaluable, which was defined as reaching week 23 after limb amputation without disease progression. Given the susceptibility of this analysis to bias, the results of the per-protocol analysis are presented as Supplementary Data (Supplementary Table S2).

Results

Patient demographics

Of the 324 dogs that were enrolled and randomized, 15 were removed because of a non-osteosarcoma histologic diagnosis ($n = 8$), regional lymph node metastases ($n = 4$), or other factors ($n = 3$; **Fig. 2**). A total of 309 dogs formed the intent-to-treat population for further statistical analysis, with $n = 157$ in the SOC arm and $n = 152$ in the SOC + S arm. The median age was 8.1 years (range, 1.4–15) and the median body weight was 38.8 kg (range, 21.2–94.5), consistent with patient populations described in other studies of canine osteosarcoma. No significant differences were seen in these patient characteristics between the two arms of the trial (**Table 1**).

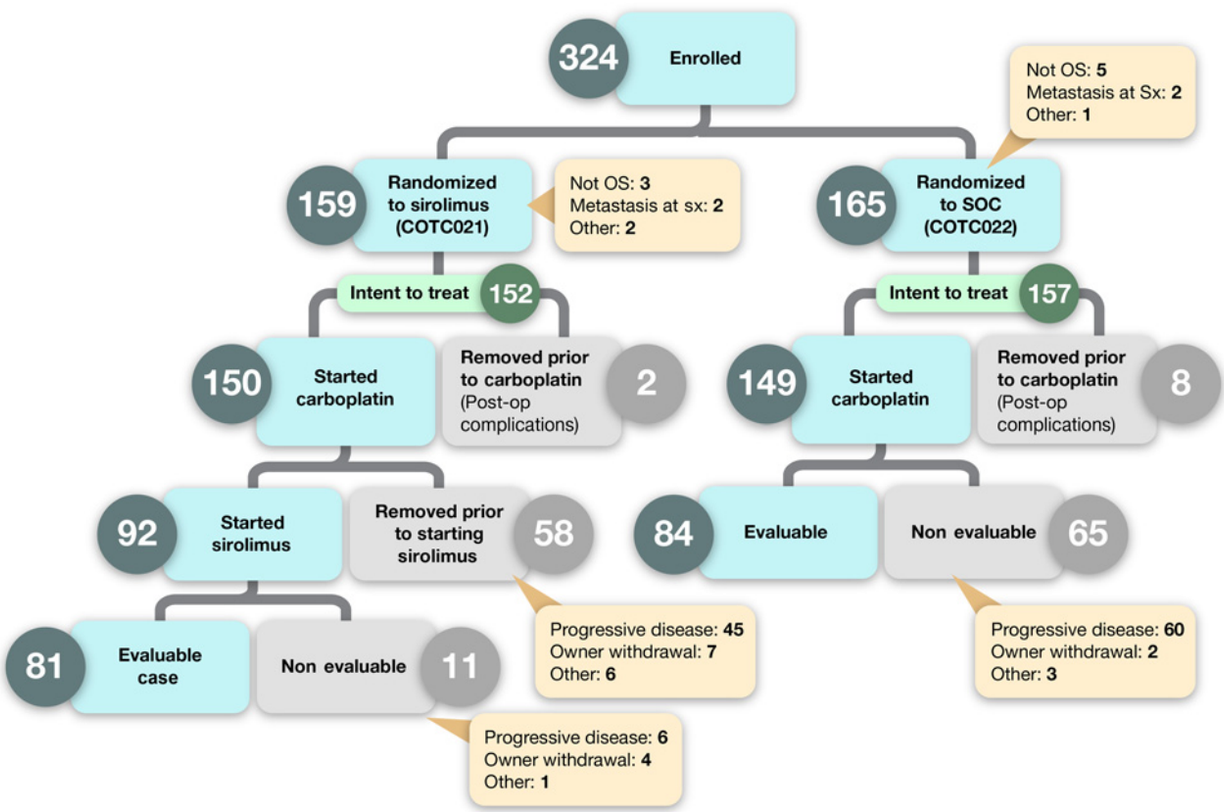

Figure 2.

Events after enrollment are captured as dogs were randomized to either SOC or SOC + sirolimus (SOC + S). Dogs that were removed from study prior to completing carboplatin chemotherapy were done so through their owners' wishes. Sx, surgery.

Table 1. Demographic features for pet dogs enrolled in the SOC or SOC + sirolimus (SOC + S) clinical trial arms.

	SOC	SOC + S
Intent-to-treat population	157	152
Age in years (median, range)	8.3 (1.4–15.6)	7.9 (1.5–13)
Weight (kg; median, range)	38.8 (25–94.5)	39.0 (21.1–75.8)
Sex		
Castrated male	83 (53%)	90 (59%)
Spayed female	64 (41%)	55 (36%)
Intact male	7 (4.5%)	6 (4%)
Intact female	3 (1.5%)	1 (1%)
ALP status		
Normal	118 (75%)	117 (77%)
Elevated	39 (25%)	35 (23%)
Tumor location		
Proximal humerus	33 (21%)	30 (20%)
Nonproximal humerus	124 (79%)	122 (80%)
Distal radius	57	53
Distal tibia	27	22
Distal femur	19	27
Proximal tibia	9	9
Ulnar	4	5
Other	8	6

Note: ALP refers to total serum ALP activity, indicated as normal or elevated based upon each COTC institution's clinical pathology laboratory reference interval.

Carboplatin chemotherapy dose reductions and/or delays

Of the 309 dogs in the intent-to-treat population, 299 (97%) dogs [SOC ($n = 149$) and SOC + S ($n = 150$)] started carboplatin therapy within 21 days of surgical amputation (**Table 2**). Ten dogs [SOC ($n = 8$) and SOC + S ($n = 2$)] were removed from study prior to chemotherapy due to post-operative complications. Thirteen (4%) dogs [SOC ($n = 7$) and SOC + S ($n = 6$)] had both carboplatin-attributable dose delays and dose reductions. Six (2%) dogs [SOC ($n = 1$) and SOC + S ($n = 5$)] had a dose reduction. A total of 141 (47%) dogs [SOC ($n = 75$) and SOC + S ($n = 66$)] had a dose delay defined as an intertreatment carboplatin dosing interval of longer than 21 days. Carboplatin-attributable dose reductions and delays were because of drug-associated neutropenia and thrombocytopenia. The remaining 139 (46%) dogs [SOC ($n = 66$) and SOC + S ($n = 73$)] received carboplatin per study protocol without deviation.

Sirolimus therapy

Walk-in tolerability

Oral sirolimus was tolerated in the majority of 22 tumor-bearing dogs receiving a dose of 0.1 mg/kg daily given either M–F or M/W/F schedules with weekends off for 4 consecutive weeks. However, a minority (20%) of patients demonstrated reduced appetite and gastrointestinal upset during the last 2 weeks of study, which was likely attributed to heavy disease burden and constitutional compromise. As drug tolerability was the endpoint, clinical response to sirolimus therapy was not assessed in the walk-in trial.

Walk-in pharmacokinetics

Sirolimus administered at 0.1 mg/kg orally on a M–F schedule for 26 days provided an AUC/dose equivalent exposure of 4,367 [ng*hour/mL]/[mg/kg] (Supplementary Fig. S2A), while the same dose administered on a M/W/F schedule produced lower AUC/dose equivalent exposures (Supplementary Fig. 2B). Pharmacokinetic modeling based on these walk-in data was used to simulate a 4-day on/3-day off schedule (M–Th; **Fig. 3A**, shaded curves). Simulation to include a 3-day weekend drug holiday was performed to address potential issues with tolerability of sirolimus when administered chronically (e.g., a planned treatment interval longer than 4 weeks). The AUC/dose equivalent from this simulation was 2,403 ± 2,246 [ng*hour/mL]/[mg/kg]. These actual and simulated values exhibit significant variability, yet compare favorably with the AUC/dose equivalent of 3,555 [ng*hour/mL]/[mg/kg] predicted to exert an antimetastatic effect in mouse models of osteosarcoma (14). However, this simulation did not predict achievement of trough sirolimus concentrations approximating 10–15 ng/mL, which has been held as a pharmacokinetic target for solid tumor studies in pediatric patients receiving sirolimus treatment (28, 29).

Adjuvant SOC + S arm tolerability

Ninety-two (64%) dogs started sirolimus treatment at week 15. Sixty-four dogs (70%) completed all four cycles, 5 (5%) completed three cycles, 12 (13%) completed two cycles, 7 (8%) completed one cycle, and 4 (4%) did not complete the first cycle. Of the 11 dogs that did not complete two cycles of sirolimus, 6 dogs were removed due to disease progression, 4 dogs due to owner removal, and 1 dog due to a concurrent complicating illness. One dog underwent a sirolimus dose reduction, 7-day drug holiday, and a schedule modification to M/W/F dosing due to adverse events.

Adjuvant SOC + S arm pharmacokinetics

Pharmacokinetic data from cycle 1-day 11 and cycle 1-day 25 from $n = 61$ dogs treated M–Th in SOC + S arm (**Fig. 3A**, red dots) were compared with the simulations of M–Th dosing generated within the walk-in cohort of dogs (**Fig. 3A**, shaded curves). These results show that the measured data (depicted by red dots at pharmacokinetic collection timepoints on cycle 1-day 11 and -day 25) are widely distributed across the simulated exposure curve. Furthermore, the majority of dogs demonstrated measured sirolimus blood levels below 10 ng/mL. The AUC/dose equivalents for cycle 1-day 11 and cycle 1-day 25 timepoints were 1,499 ± 1,446 [ng*hour/mL]/[mg/kg] and 1,357 ± 1,475 [ng*hour/ml]/[mg/kg], respectively. To assess sirolimus pharmacokinetic variability across treatment duration (cycles 1–4), sirolimus levels were measured at 2- and 8-hours post-dosing (**Fig. 3B**). ANOVA showed that 2- and 8-hour timepoints were significantly different ($P < 0.0001$), but none of these average values were significantly different across cycles of treatment ($P = 0.4825$). Within this dataset, intrapatient variability was relatively small compared with interpatient variability, suggesting that pharmacokinetic differences were largely patient dependent. The magnitude of interpatient pharmacokinetic variability was underscored by the wide range trough concentrations calculated from a subset of dogs ($n = 78$) receiving sirolimus 0.53–30.98 ng/mL, which span across predicted subtherapeutic (<10 ng/mL) and therapeutic (>10 ng/mL) concentrations.

AE reconciliation: severity and attribution

The most common recorded AEs attributable to surgery were pain ($n = 33$ dogs) and surgical site seromas ($n = 22$ dogs). Carboplatin produced self-limiting AEs, including neutropenia ($n = 261$), thrombocytopenia ($n = 178$), anorexia ($n = 46$), diarrhea ($n = 36$), and vomiting ($n = 33$). There were no grade 5 events (fatal) attributable to carboplatin. Sirolimus exposure at 0.1 mg/kg was well tolerated, with only 17 (18%) of the 92 dogs experiencing events, and the most common adverse clinical symptoms being lethargy/fatigue ($n = 7$),

Table 2. Clinical outcome measures for dogs within the intent-to-treat population, enrolled in SOC and SOC + S trial arms.

		SOC	SOC + S
Intent-to-treat population		157	152
	Started SOC	149 (95%)	150 (99%)
	Completed SOC	114 (73%)	112 (74%)
	Started sirolimus treatment	N/A	92 (61%)
	Completed sirolimus treatment	N/A	64 (42%)
Reason off-study			
	Disease progression on study	52 (33%)	83 (55%)
	Disease progression during follow-up period	69 (44%)	34 (22%)
	Complicating disease/intercurrent illness	13 (8%)	8 (5%)
	Follow-up period completed	9 (6%)	7 (5%)
	Refused further treatment	3 (2%)	12 (8%)
	Refused further follow-up	3 (2%)	4 (3%)
	Death on study	2 (1%)	3 (2%)
	Death during follow-up period	3 (2%)	0 (0%)
	AEs/side effects	3 (2%)	1 (<1%)
Dogs dead during the study period		146 (93%)	143 (94%)
	Dead with evidence of metastatic disease[a]	118 (75%)	124 (82%)
	Dead without evidence of metastatic disease	28 (18%)	19 (13%)
Dogs alive at the end of the follow-up period		6 (4%)	9 (6%)
	Alive with evidence of metastatic disease	0 (0%)	1 (<1%)
	Alive without evidence of metastatic disease	6 (4%)	8 (5%)
Dogs lost to follow-up		5 (3%)	8 (5%)

Abbreviations: AE, adverse events; N/A: not applicable.
[a]Clinical, imaging, or necropsy evidence.

diarrhea ($n = 6$), anorexia ($n = 5$), and nausea/ptyalism ($n = 4$). There were only two grade 3 events (fever), and one grade 4 event (nausea). There were no grade 5 events attributable to sirolimus. There were five deaths on study, none attributable to carboplatin or sirolimus treatment. One dog developed disseminated intravascular coagulation within 24 hours of surgery and died due to cardiopulmonary arrest 3 days after surgery. The additional three known causes of death were gastric dilatation with volvulus, endocarditis, and hemorrhage secondary to gastrointestinal ulcer perforation. An additional dog died at home due to unknown causes prior to starting carboplatin.

Clinical outcomes

Figure 2 depicts the progress of all 324 cases that were enrolled, and, of these, the 309 cases comprised the intent-to-treat population from enrollment to evaluable/nonevaluable status. In order for a case to be deemed fully evaluable, dogs enrolled to the SOC arm had to complete carboplatin therapy and be deemed free of metastatic disease at the week 23 visit. For the SOC + S arm, dogs had to complete carboplatin, be deemed free of metastatic disease at week 15, and then complete two cycles of adjuvant sirolimus therapy (week 23). Within the intent-to-treat population, the DFI (180 vs. 204 days; $P = 0.87$) and overall survival (282 vs. 280 days; $P = 0.98$) were not significantly different between the SOC and SOC + S groups (**Fig. 4**).

SOC

The median DFI for this group was 180 days (95% CI, 144–237), with 27.5% and 14.3% of dogs disease free at 1 and 2 years post-diagnosis, respectively (**Fig. 4**; Supplementary Table S3). The median overall survival was 282 days (95% CI, 224–383), with 43.9% and 24.8% of dogs alive at 1 and 2 years post-diagnosis, respectively. No differences were appreciated within the subgroups based on ALP status and tumor location within the SOC arm.

SOC + S

The median DFI for this group was 204 days (95% CI, 157–217), with 26.7% and 15.5% of dogs disease free at 1 and 2 years post-diagnosis, respectively (**Fig. 4**; Supplementary Table S4). The median overall survival was 280 days (95% CI, 252–332), with 41.3% and 23% of dogs alive at 1 and 2 years post-diagnosis, respectively. In contrast to the SOC arm, significant differences were identified between the subgroups represented by tumor location and ALP status. Within the assessment of DFI among subgroups, dogs with a nonproximal humeral tumor and normal ALP achieved the longest DFI (210 days), while dogs with a proximal humeral tumor and elevated ALP had the shortest DFI (131 days). This same pattern was not discernable in the overall survival analysis of the entire study arm inclusive of all four subgroupings, with the longest survival seen in dogs with a nonproximal humeral tumor and a normal ALP (320 days) and shortest in dogs with a nonproximal humeral tumor and an elevated ALP (203 days).

Disease progression and correlations with sirolimus pharmacokinetics

Metastatic pattern

Necropsy was performed in 42 and 41 dogs in SOC (27%) and SOC + S (27%) arms, respectively. Upon necropsy, 56 dogs (67%) were found to have disease in multiple sites, with the most common sites being lung, liver, kidney, heart, ribs, bone, and spine. Dogs that did not undergo a necropsy and had metastatic disease confirmed on the basis of physical examination and radiographs, distant lesions were most often identified involving the lung (173 dogs) or bone (18 dogs).

Pharmacokinetic outcome association

No association was found between mean AUC on DFI (HR, 1.000; 95% CI, 0.998–1.003; $P = 0.76$) or overall survival time (HR, 1.000; 95% CI, 0.998–1.003). In addition, no associations were found with development of grade 3 or worse AEs and DFI (HR, 2.343; 95% CI,

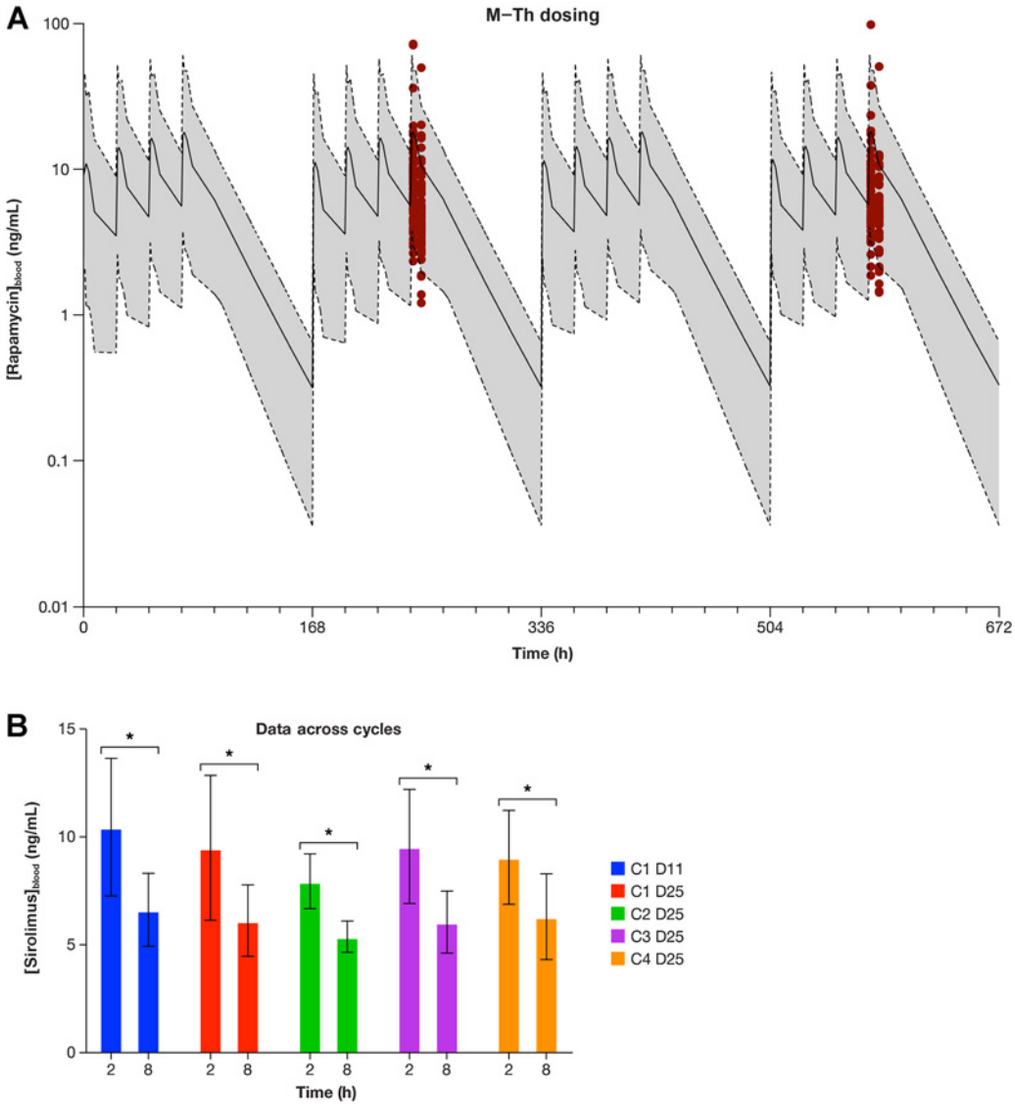

Figure 3.

A, Red dots depict the sirolimus blood levels that were measured in dogs receiving 0.1 mg/kg orally on a M–Th basis within the SOC + S trial. These data were generated from whole blood of dogs ($n = 61$) on cycle 1 day 11 and cycle 1 day 25, and are overlaid with the simulated AUC that was predicted from a simulation of M–Th dosing of 0.1 mg/kg (solid line, simulated mean sirolimus concentration; shaded area bound by dotted lines, simulated range of sirolimus concentration). The simulated sirolimus AUC data were generated from the walk-in study of sirolimus. **B,** Sirolimus concentrations in whole blood shown as the average value and 95% CI, obtained from dogs receiving 0.1 mg/kg of sirolimus orally within the SOC + S clinical trial, at the 2- and 8-hour timepoints across the four cycles of drug treatment (C1 D11, cycle 1 day 11; C1 D25, cycle 1 day 25; C2 D25, cycle 2 day 25; C3 D25, cycle 3 day 25; C4 D25, cycle 4 day 25). ANOVA shows that the 2- and 8-hour timepoints for each cycle are significantly different (*, significant difference, $P < 0.0001$), but none of the 2- or 8-hour average values were significantly different ($P = 0.4825$) when compared across dosing cycles.

0.927–5.920; $P = 0.07$) or overall survival time (HR, 1.496; 95% CI, 0.597–3.751; $P = 0.39$). However, this is likely due to the low number of sirolimus attributable grade 3 events ($n = 2$; fever), and one grade 4 event (nausea).

Discussion

This clinical trial illustrates a convergence research approach, whereby a cooperative comparative oncology network (NCI-COTC) under the umbrella of the Osteosarcoma Project was able to rapidly generate biologically rich data within a naturally occurring disease model, such as the pet dog, which permits translational hypothesis testing to be explored in a mammalian species that more accurately recapitulates the natural history and progression of human osteosarcoma. The results of this clinical trial showed that adjuvant sirolimus therapy administered sequentially following amputation and chemotherapy in dogs with appendicular osteosarcoma was clinically tolerable, yet did not significantly improve DFI or survival over amputation and chemotherapy alone. While the absence of effect observed in this translational study was disappointing and discordant with existing preclinical metastatic osteosarcoma mouse models (14, 15), the clinical outcomes derived from this prospective clinical trial and associated

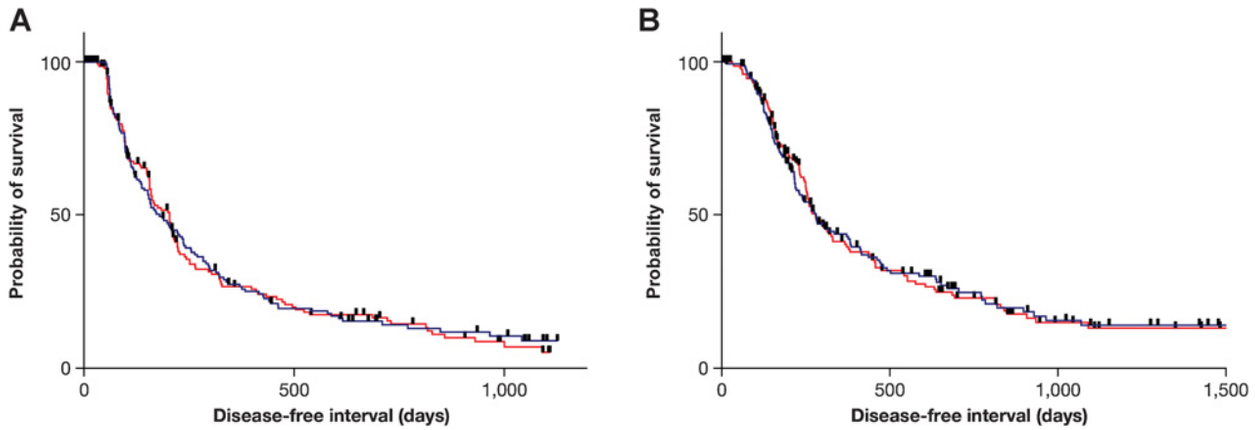

Figure 4.

Kaplan–Meier event curves depicting the probability of survival with respect to DFI (**A**) and overall survival (**B**) between the SOC (blue line) and SOC + S (red line) arms of the trial.

study design should be viewed with compelling credibility. Given the abundance of scientific and clinical data supporting the intrinsic value of pet dogs with naturally occurring osteosarcoma to serve as sophisticated models for recapitulating the pathology of the human disease (2, 11), the findings described in this study should be leveraged to more fully understand the therapeutic framework and constraints associated with mTOR inhibition, as well as to promote the guidance for how PI3K/mTOR vulnerabilities can be more optimally targeted for curbing metastatic osteosarcoma progression.

Signaling through PI3K/mTOR has been identified as a major oncogenic driver conserved across divergent tumor histologies (30), and substantive data support this pathway to be frequently hyperactivated in both human and canine osteosarcoma through various genomic changes, including activating mutations in *PI3K*, amplifications of *AKT*, and *PTEN* downregulation (13, 31–33). Collectively, these genomic perturbations exert pleiotropic protumorigenic effects, including osteosarcoma invasion, cell-cycle dysregulation, apoptosis evasion, angiogenesis, chemoresistance, and metastasis (34, 35). Given its putative role in osteosarcoma progression, inhibition of PI3K/mTOR signaling has been identified as a conserved therapeutic vulnerability for osteosarcoma (12, 13), and a large body of *in vitro* studies and preclinical metastatic osteosarcoma mouse models have substantiated these genomically identified oncogenic susceptibilities when targeted by mTOR inhibitors, including sirolimus (14–16, 22, 36, 37).

Although existing genomic and biologic data strongly underscore the role of PI3K/mTOR signaling in osteosarcoma progression, translation of these scientific and preclinical findings has largely remained unrealized for improving the clinical management of metastatic bone sarcomas. Despite multiple clinical reports describing the alluring potential of mTOR inhibition strategies for stabilizing or even regressing macroscopic recurrent or metastatic osteosarcoma lesions using sirolimus (19) or rapalogs (38–40), large randomized studies evaluating mTOR inhibition strategies to impede micrometastatic osteosarcoma disease progression have not been systematically investigated in the context of phase III human clinical trials. To date the most significant study which partially addresses this clinical gap in knowledge regarding the antitumor potential of mTOR inhibitors is the SUCCEED trial, an international randomized phase III trial that evaluated the ability of ridaforolimus, a non-prodrug analogue of sirolimus, to maintain benefits from prior cytotoxic chemotherapy for the prolongation of disease stability in patients with advanced sarcomas. In total, 711 patients with advanced sarcomas [soft tissue (n = 642) and bone (n = 69)] were enrolled, including a subgroup of 50 patients with osteosarcoma (1:1 ridaforolimus to placebo). Whereas ridaforolimus demonstrated statistically significant improvements in progression-free survival versus placebo (17.7 vs. 14.6 weeks, respectively) for the entire study population, subgroup analysis of patients with osteosarcoma was not adequately powered to detect any differences between treatment groups, but improvement in progression-free survival for the ridaforolimus cohort was not detected in the limited osteosarcoma population evaluated (n = 50; ref. 41). Unfortunately, while ridaforolimus demonstrated statistically significant activity, the magnitude of clinical benefit was not sufficient to warrant new drug approval designation for the management of metastatic soft-tissue or bone sarcomas.

While the SUCCEED trial identified constraints of ridaforolimus in patients with osteosarcoma presenting with recurrent or relapsed disease, the translational potential of mTOR inhibition for thwarting micrometastatic osteosarcoma progression has largely remained unanswered and predominantly limited to preclinical investigations reliant upon human xenograft mouse studies (14, 15). Through the inclusion of pet dogs with naturally occurring osteosarcoma, this study was uniquely suited to provide high-value answers to this clinical gap in knowledge regarding the clinical impact of mTOR inhibition on osteosarcoma micrometastatic progression. In this study, the longitudinal outcomes in pet dogs failed to demonstrate any measurable effect on micrometastatic disease progression despite mTOR inhibition achieved by oral sirolimus when sequentially administered, following the completion of systemic chemotherapies. Significant differences in progression-free and overall survival were not identified in dogs treated with or without sirolimus, and these findings do not support the inclusion of oral sirolimus sequentially following SOC therapy (amputation + chemotherapy) for slowing micrometastatic progression in canine osteosarcoma. Despite the negative clinical findings associated with sirolimus intervention, this trial has allowed for the amassment of high-value biologic samples, which can be leveraged for ongoing and future studies to deeply study the mechanisms and vulnerabilities of osteosarcoma metastatic progression.

Whereas the lack of improved progression-free and overall survival time in dogs treated with sirolimus was disappointing, any limitations of mTOR inhibition for delaying osteosarcoma micrometastatic disease progression identified in this study should be viewed contextually through the lens of disease biology, pharmacokinetics, and clinical trial design. First, while PI3K/mTOR has been identified as a therapeutic vulnerability for osteosarcoma (12, 13), given the genomic heterogeneity of osteosarcoma, it would be erroneous to assume that broad clinical benefit should be expected from a pan-mTOR inhibition strategy. Rather, recent scientific investigations strongly point toward a genome-informed targeted therapy approach for osteosarcoma, whereby molecular subtypes of osteosarcoma would be vulnerable to specific single or combination inhibition strategies (12, 42). In the absence of genomic/molecular subtyping, pan-mTOR inhibition strategies might only benefit a minority of human or canine patients treated (13, 33), and any positive treatment effects achieved in a small percentage of individuals could become indiscernible with aggregated data analysis. Second, in this study, dogs were administered sirolimus at a biologically effective dose (17, 18) sufficient to inhibit mTOR signaling at 0.1 mg/kg M–Th schedule for up to four cycles (26 days/cycle). Despite historical studies demonstrating modulation of downstream mTOR targets (pS6RP/S6RP) with similar dosing strategies, trough concentrations of sirolimus in dogs predominantly ranged from 1 to 10 ng/mL, being slightly lower than target sirolimus concentrations in humans that exert immunosuppressive and anticancer activities (5–15 ng/mL; refs. 28, 29, 43). In contrast, in mouse models of osteosarcoma whereby sirolimus either attenuates heterotopic primary tumor growth (16) or delays orthotopic, spontaneous pulmonary metastases progression (14, 15), biologically active trough concentrations of sirolimus have been approximately 10-fold greater (14). Based upon the relatively low trough concentrations of sirolimus (<10 ng/mL) achieved in the majority of dogs enrolled in this study, it remains a distinct possibility that insufficient drug exposure (concentration) or duration of exposure (maximum four cycles) might have contributed to the absence of antimetastatic activities in pet dogs receiving sirolimus. Finally, prevailing evidence tightly links enhanced mRNA translational efficiency with successful osteosarcoma metastasis biology (14), with activation of the mTOR signaling pathway and protein translational efficiency most critical during periods of cellular stress encountered along the metastatic cascade continuum, but likely accentuated during the most inefficient steps of metastasis, that is, colonization (44). Given the importance of translational efficiency for successful metastasis, inhibition of the mTOR pathway would be expected to be most effective during periods when cancer cells are subjected to biological stressors (endogenous or exogenous). In this trial, dogs were treated sequentially with SOC (amputation and chemotherapy) and then sirolimus, a purposeful clinical trial design to minimize undesirable hematologic toxicities associated with contemporaneous chemotherapy and mTOR inhibition strategies (45). However, the delayed introduction of sirolimus into the treatment protocol of dogs at week 15 might have resulted in a "too little too late" effect, and it is reasonable to speculate that exposure to sirolimus, and consequent mTOR inhibition, would be more successful when administered concurrently with exogenous biologically stressors, such as chemotherapy. As such, concurrent and combinatorial strategies, which temporally couple mTOR inhibitors with other therapies as employed in human clinical trials, would be predicted to have the greatest impact for improving the management of osteosarcoma metastatic progression. Similarly, sirolimus exposure early in the natural disease course during active cancer cell colonization, as practiced in the generation of metastatic mouse models of osteosarcoma (14, 15), would be expected to increase the likelihood for observing beneficial antimetastatic activities.

Although not performed as a component of this trial, molecular profiling of canine osteosarcoma is critical to understand in what ways the canine disease recapitulates the genomic framework of human osteosarcoma. Efforts are currently underway to characterize the samples obtained from dogs enrolled in this trial to identify correlative genomic subtypes within dog and human patients with osteosarcoma, explore tumor clonality and genomic evolution, and devise a model of platinum-based chemotherapy resistance in canine osteosarcoma. These data will help to further support the basis for including the pet dog with osteosarcoma as a naturally occurring model for human osteosarcoma, building upon examples from the literature and supporting specific selection of canine osteosarcoma subpopulations to participate in studies of molecularly targeted agents (9, 46).

Metastatic progression continues to be the life-limiting event for both human patients and canine patients with osteosarcoma, despite ongoing efforts to deconvolute the complex biology of osteosarcoma and conduct clinical trials of novel agents in both species. The spontaneous development of osteosarcoma in an immunocompetent pet dog, coupled with the rapidity of disease progression, offers several advantages for osteosarcoma drug development inclusive of immunotherapies (46–48). Given that the majority (∼90%) of dogs will succumb to osteosarcoma progression and disease-related death within months to 2 years of diagnosis, the rapid conductance of phase III–like clinical trials, as the one described herein, can generate mature results within 3–5 years. Indeed, the outcomes for dogs receiving SOC therapy in this trial are consistent with other prior studies (9, 46). This deeply annotated clinical dataset provides an opportunity for data sharing efforts that enable comparison of novel adjuvant treatment strategies assessed in future trials. The experience gained through the conduct of this trial provides several opportunities to reflect on how to improve and optimize the design and implementation of future studies. Among these are the ability to pivot toward shorter observational periods occurring immediately after amputation, to emphasize improvement upon early failure rates, and a greater emphasis placed on combinatorial drug strategies in both the minimal residual disease and gross metastatic disease setting. As such, the inclusion of pet dogs as a unique model for pediatric osteosarcoma can synergize and complement ongoing efforts which utilize rapid phase II trial designs for the identification of the most promising agents to quickly advance toward upfront phase III clinical trials (49, 50).

Authors' Disclosures

No disclosures were reported.

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