

Phase I Clinical Pharmacology Study of F14512, a New Polyamine-Vectorized Anticancer Drug, in Naturally Occurring Canine Lymphoma

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Abstract

Purpose: F14512 is a new topoisomerase II inhibitor containing a spermine moiety that facilitates selective uptake by tumor cells and increases topoisomerase II poisoning. F14512 is currently in a phase I/II clinical trial in patients with acute myeloid leukemia. The aim of this study was to investigate F14512 potential in a new clinical indication. Because of the many similarities between human and dog lymphomas, we sought to determine the tolerance, efficacy, pharmacokinetic/pharmacodynamic (PK/PD) relationship of F14512 in this indication, and potential biomarkers that could be translated into human trials.

Experimental Design: Twenty-three dogs with stage III–IV naturally occurring lymphomas were enrolled in the phase I dose-escalation trial, which consisted of three cycles of F14512 i.v. injections. Endpoints included safety and therapeutic efficacy.

Serial blood samples and tumor biopsies were obtained for PK/PD and biomarker studies.

Results: Five dose levels were evaluated to determine the recommended dose. F14512 was well tolerated, with the expected dose-dependent hematologic toxicity. F14512 induced an early decrease of tumoral lymph node cells, and a high response rate of 91% (21/23) with 10 complete responses, 11 partial responses, 1 stable disease, and 1 progressive disease. Phosphorylation of histone H2AX was studied as a potential PD biomarker of F14512.

Conclusions: This trial demonstrated that F14512 can be safely administered to dogs with lymphoma resulting in strong therapeutic efficacy. Additional evaluation of F14512 is needed to compare its efficacy with standards of care in dogs, and to translate biomarker and efficacy findings into clinical trials in humans. *Clin Cancer Res*; 1–10. ©2015 AACR.

Introduction

Non-Hodgkin lymphoma is the seventh most common human systemic malignancy, with an estimated prevalence of 70,000 patients in the United States in 2013 (1). The addition of anti-CD20 therapy to the multidrug chemotherapy regimen CHOP (cyclophosphamide–adriamycin–vincristine–prednisolone) greatly improved the prognosis of diffuse large B-cell lymphoma (DLBCL), but nearly one third of patients relapsed, underlining the considerable possibility for therapeutic improvement (2).

Vectorization is an ingenious way to improve tumor selectivity of known therapeutic agents, by conjugating them with a chemical entity to target cancer cells more specifically. One possibility is to exploit a selective transport system, such as the polyamine transport system, which is overactive in many tumor cells (3). The anticancer drug candidate F14512 is designed to target cancer cells through the polyamine transport system. It contains a spermine chain in place of the C4 glycosidic moiety of etoposide (4). The positively charged spermine tail contributes to (i) favoring the selective uptake of the drug by tumor cells via the polyamine transport system, (ii) increasing DNA binding to reinforce topoisomerase II inhibition, (iii) enhancing the water solubility of the drug. These properties translated into a favorable pharmacologic profile: F14512 has demonstrated potent *in vitro* and *in vivo* antitumor activities in preclinical studies, and shown to be superior to etoposide, its parent compound (4–11).

With the support of this solid preclinical data, F14512 progressed to clinical development and a phase I clinical trial was initiated in refractory/relapsing acute myeloid leukemia (AML). Promising antileukemic activity was observed at different dose levels (12) and F14512 is currently in a phase I/II trial in AML in combination with cytarabine.

Preclinical data showed that lymphoma could be an indication of interest for F14512 development (4). Dogs may be the most relevant animal model to study new therapies for this indication. Actually, naturally occurring lymphomas in dogs are closer to their human counterparts than any xenograft mice models,

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

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doi: 10.1158/1078-0432.CCR-14-3174

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Translational Relevance

Despite the approval over the past 10 years of several targeted therapeutic drugs, it is important to continue to test cytotoxic agents that still play a major role in high-grade lymphoma therapy. Drugs that are specifically vectorized to cancer cells, such as the new polyamine-vectorized drug F14512, should offer reinforced activity to tackle tumor cells while sparing normal cells, resulting in an improved therapeutic index and/or reducing unwanted toxicities. Although traditional preclinical mice models often fail to accurately predict the antitumor activity and toxicity of new therapies, comparative oncology has revealed that spontaneous lymphomas in dogs share clinical, biologic, genetic, and therapeutic similarities with their human counterparts. This study showed the therapeutic relevance of the vectorized drug F14512 in a canine lymphoma model and has potential translational relevance for both the ongoing clinical development of F14512 and the treatment of lymphomas in humans.

because of their growth over long periods of time in an intact immune system, interindividual and intratumoral heterogeneity, development of recurrent or resistant diseases, and metastasis (13). Clinical presentation, biologic behavior, tumor genetics, and treatment response are very similar between canine and human DLBCL (14, 15). Moreover, recent gene profiling expression studies emphasized the molecular similarities of DLBCL in both species (16, 17). Finally, the relevance of dogs as a lymphoma model is supported by their use in clinical trials (18, 19).

Etoposide has been extensively studied in dogs for pharmacokinetics (PK) and toxicologic purposes (20, 21), but little data are available concerning its potential antitumor efficacy in pet dogs (22, 23). A retrospective study on 13 dogs with relapsing lymphoma treated with etoposide (24) showed that etoposide had a minimal therapeutic effect. Etoposide phosphate administration in dogs was associated with hematologic toxicity (20). An acute pruritic cutaneous reaction was also observed, but it could have been linked to the vehicle used in this study (24). As etoposide had poor results in a pet dog lymphoma model, it was of great interest to evaluate F14512, a vectorized and more soluble form of etoposide, in this model to validate the proof of concept and the benefits of this tumor-targeting agent.

The aims of this dose-escalation clinical trial were therefore (i) to assess the potential clinical and hematologic tolerance and to determine the MTD of F14512 in a population of dogs with naturally occurring lymphoma, (ii) to describe the PK characteristics of F14512 in these dogs, and (iii) to identify early signs of efficacy of this treatment, with an evaluation of the remission rate after three cycles of therapy. This was a translational research study, as PK/pharmacodynamic (PD) relationships, biomarker, and efficacy can be directly translated into ongoing and future clinical trials of F14512.

Materials and Methods

Chemicals

F14512 was provided by Pierre Fabre Medicament. The design and synthesis of F14512 have been patented (WO 2005/100363).

Cell culture

Namalwa (Burkitt's lymphoma—ATCC CRL-1432) cells were grown in RPMI-1640 medium with 10% FCS, 2 mmol/L L-glutamine, 100 µg/mL penicillin–streptomycin, and 1.25 µg/mL fungizone. Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ and maintained using standard cell culture techniques.

Antiproliferative activity

Namalwa cells were seeded in 96-well plates, treated with increasing concentrations of F14512 and incubated for 72 hours. Cell viability was then evaluated by dosing the ATP released by viable cells using the ATPlite assay (PerkinElmer). EC₅₀ values were determined with a curve-fitting analysis (a nonlinear regression model with a sigmoidal dose response, variable hill slope coefficient), performed with the GraphPad Software algorithm.

Cell-cycle analysis

Namalwa cells were seeded in 96-well plates and incubated with 6 different concentrations ranging from 0.125- to 5-fold the IC₅₀ value (determined in the ATPlite assay) for 24, 48, and 72 hours. Cells were labeled with the Kit Coulter DNA-Prep Reagents (Beckman Coulter) and analyzed with a Guava PCA-96 flow cytometer (Merck Millipore). The percentage of cells in each cell-cycle phase was calculated using M Cycle software.

DNA double-strand breaks detection

Namalwa cells were seeded in 12-well plates and exposed to increasing concentrations of F14512 for 4, 16, and 24 hours. To detect DNA double-strand breaks (DSB), cells were fixed and permeabilized with DNA-prep LPr reagent (Beckman Coulter) and stained with an Alexa Fluor 647 anti-H2AX-phosphorylated (ser139) antibody (Biolegend). For the DNA content analysis, cells were stained with DNA-prep STAIN reagent. Phospho-H2AX-positive cells were analyzed on a LSR-II cytometer (BD Biosciences).

Annexin V–PE/Nexin-7AAD staining experiments

Namalwa cells were seeded in 96-well plates and treated with increasing concentrations of tested compound for 16 hours. The cells were then trypsinized and stained with the Guava PCA-96 Nexin Kit (Guava Technologies). This kit contained Annexin V combined with phycoerythrin and 7-amino actinomycin D (7-AAD) that bound to the intracellular nucleic acid when the membrane integrity was impaired. Thereafter, samples were analyzed with a Guava PCA-96 flow cytometer.

Caspases 3/7 activation measurements

Namalwa cells were seeded in 96-well plates, treated with different concentrations of F14512 and incubated for 16 hours. Caspases 3/7 enzyme activities were measured using the Caspase-Glo 3/7 assay (Promega Corp.), in accordance with the manufacturer's instructions.

Fine-needle aspirates

Fine-needle aspirates were performed by a clinical trial and veterinary care assistant using 21-gauge needles. Cells were collected from three different lymph nodes on which an average of three punctures had been performed. Aspirates were then flushed in a 4 mL vial containing PBS (adapted from refs. 25 and 26). The needle was redirected several times during aspiration to avoid cell

collection from the same area. Subsequent sample collections were carried out on the same lymph nodes used for the initial diagnosis and evaluation (as described by Williams and colleagues; ref. 27). A cell count was performed twice on each sample using Nexcelom Cellometer Auto T4 (Ozyme Biosciences) and Covalab cell chambers.

Ex vivo P-H2AX cytometry analysis

Phospho-H2AX levels were quantified using the flow-cytometry technique adapted from Huang and colleagues (28). Experiments were performed on fine-needle aspirate samples. Cells were fixed in a 2% formalin solution and permeabilized in a 70% cold ethanol solution then washed and rehydrated in PBS, 4% FCS and 0.1% Triton X-100. Nuclear H2AX staining was performed using a rabbit anti canine-gamma-H2AX antibody (NB100-384; Novus biologicals) and a secondary antibody (Cy5-goat anti-rabbit IgG A10931; Invitrogen Life Technologies).

Dog selection

This study was conducted by Oncovet Clinical Research (OCR) as part of a collaborative research project between OCR and Pierre Fabre Medicament. As the drug provider, Pierre Fabre Medicament also provided technical and scientific assistance. The trial was available for all dogs that (i) had new or previously diagnosed multicentric lymphoma; (ii) had a measurable disease at study entry (allowing diagnosis and staging) with no restriction on the stage of the disease; (iii) had not responded to standard therapies (including chemotherapy and/or glucocorticoids) or whose owners had declined standard therapies; (iv) had no anticancer treatment in the month before entry to the study; (v) had no significant biochemical abnormality or cytopenia, which precluded the use of cytotoxic drugs; (vi) had no concurrent serious systemic disorder incompatible with this study; (vii) had a life expectancy of at least 9 weeks, according to veterinary opinion.

Initial staging was performed on all dogs using a standardized protocol, with a histologic confirmation of diagnosis. The dogs were staged at the time of diagnosis based on the World Health Organization (WHO) classification: five-stage criteria for canine lymphoma and lymph node size were assessed using published recommendations (29). Staging tests included a complete blood count, chemistry panel, two-view chest X-rays, an abdominal ultrasound, and a bone marrow aspirate.

This study was approved by the OCR Ethical Committee. Dogs were only enrolled after the obtention of a signed informed consent from the owner and were able to withdraw from the study at any time.

Pathology

Biopsy specimens from enlarged lymph nodes were fixed in 10% neutral-buffered formalin for 48 hours and embedded in paraffin wax. Four micrometer-thick sections were stained with hematoxylin and eosin. Immunophenotyping was performed using antibodies targeting human antigens but cross-reacting with the equivalent canine antigens. An antibody targeting CD-3 was used as a pan-T marker (monoclonal mouse anti-human F7.2.38; Dako) and an antibody targeting CD-20 was used as a pan-B marker (rabbit anti-human RB-9013-P; ThermoScientific). One null cell neoplasm (negative for both CD20 and CD3) was also evaluated for PAX5 (clone 24; Cell Mark), and BLA36 (clone A27-42; Biogenex) expression (two antigens expressed by B-cell neoplasms). The original diagnosis for each case was made by one

pathologist (I. Bemelmans). A second pathologist worked independently as an expert reviewer (T. Marchal). The classification of the 23 cases was based on cellular morphology and immunophenotypes according to WHO criteria.

Assessment of hematologic and coagulation status

Blood was collected using jugular or cephalic venipuncture. Blood samples were placed into EDTA vacutainer glass tubes and heparinized blood tubes. Analyses were performed immediately. The baseline assessment included the measurement of the chemistry profile, including an ionized calcium assessment (Catalyst biochemistry analyzer, IDEXX Laboratories Inc.), a complete blood count, including platelet concentration (Procyte Hematology analyzer, IDEXX Laboratories Inc.), and D-dimers concentration using a turbidometric immunoassay (Nycocard Reader II, NYCOMED).

Treatment schedule

All the dogs involved in the study followed the same protocol over a period of 6 weeks. This consisted of a 3-hour F14512 i.v. infusion once daily for 3 consecutive days, repeated every 2 weeks (days 1–3, days 15–17, and days 22–24). The first dose level of 0.05 mg/kg and the schedule of administration were chosen following previous preclinical studies performed in beagle dogs and to be consistent with ongoing and future clinical trials in humans.

Side effect assessment and medical intervention

Toxicities were graded according to the Veterinary Cooperative Oncology Group criteria for adverse events (30). The toxicity grade assigned to each dog was based on the nadir neutrophil or platelet count, the highest documented biochemical enzyme noted and the highest grade of gastrointestinal or constitutional symptom/toxicity noted. A total of 16 interim visits were planned in the protocol (on days 1, 3, 4, 7, 9, 11, 15, 17, 18, 22, 25, 29, 31, 32, 36, and 38). At each follow-up visit, owners were questioned about signs of adverse clinical effects, daily water intake, appetite, urination, vomiting, stool consistency/frequency, energy level, mood, and exercise tolerance. A complete blood count was performed at each visit. All dogs were monitored for a minimum of 8 weeks. Dose-limiting toxicity (DLT) was defined as prolonged (>48 hours) asymptomatic grade 4 neutropenia, febrile neutropenia, grade 4 anorexia, grade 4 vomiting, grade 4 diarrhea, or death. In all cases, the maximal toxicity grade recorded during all follow-up examinations was used for the determination of the MTD. Gastrointestinal adverse events were treated with symptomatic treatments. A prophylactic broad spectrum antibiotherapy was administered in the case of severe asymptomatic neutropenia (grades 3 and 4). Dogs with febrile neutropenia were hospitalized and treated with intravenous fluids and antibiotics.

Response assessment and follow-up

The response to the drug was evaluated at each treatment session by an oncologist in accordance with previously published criteria (31). The remission status was assessed on the basis of physical examination and mandatory lymph node cytology of the remaining enlarged lymph nodes. Two weeks after the end of the protocol (day 45), all dogs underwent complete end-staging, similar to initial staging. If requested, a complementary CHOP-based chemotherapy protocol was proposed to the dog's owner.

F14512 analysis in plasma

The PK of F14512 and its active metabolite F16490 were evaluated throughout the treatment. Six blood samples were drawn on day 1 of cycle 1 (before dosing and 1.5, 3, 3.5, 5, and 6 hours after the start of the 3-hour infusion). Several samples were also obtained during and after cycles 2 and 3. Plasma concentrations of F14512 and F16490 were quantified using a validated LC/MS-MS method with a lower limit of quantification (LLOQ) of 0.25 ng/mL.

Statistical analysis

Data are expressed either as mean \pm SD or as percentages. The primary endpoint of this study was to determine the clinical response rate (stable disease, complete or partial remission) on day 45. The selected level of significance was set at $P < 0.05$.

Results

F14512 effects in lymphoma cell lines

We previously found that F14512 (see structure in Supplementary Fig. S1A) displayed strong efficacy in a human lymphoma cell line, both *in vitro* and *in vivo* in a mouse model (4, 8). In this study, we further analyzed the effect of F14512 on the Burkitt's lymphoma cell line Namalwa. This drug induced a dose-dependent growth inhibition after a 72-hour treatment (Supplementary Fig. S1B) with a calculated EC_{50} value of 46 nmol/L (95% confidence interval; 35–61 nmol/L). We observed that Namalwa cells treated with F14512 were blocked in the G₂-M phase in a dose-dependent manner (55% and 62% after a 48-hour and 72-hour treatment, respectively, at 2 EC_{50} ; Supplementary Fig. S1C). The induction of apoptosis was first evaluated via the conventional annexin V-PE/7-AAD staining procedure. F14512 showed no effect on Namalwa cells after a 16-hour treatment at doses up to 10 μ mol/L (Supplementary Fig. S1D). These results were confirmed by measuring caspases 3 and 7 activities after exposure to the drug for 16 hours. F14512 only induced a dose-dependent increase of caspases-3/7 activity at high doses (above 3 μ mol/L; Supplementary Fig. S1E), underlining that F14512 did not behave as a proapoptotic agent.

A key step of the mechanism of action of F14512 is the topoisomerase II-dependent generation of DNA damages. Inhibition of topoisomerase II leads to the generation of cleavable complexes and DSB of DNA that are characterized by phosphorylation of histone H2AX on Ser-139. P-H2AX is used as a reporter of DNA damage.

We then evaluated the impact of F14512 on DNA damage by measuring the phosphorylation of histone H2AX on Ser-139 in Namalwa cells. F14512 induced DNA-damage in a dose- and time-dependent manner (Supplementary Fig. S2). P-H2AX increased as early as 4 hours after F14512 incubation and the percentage of stained cells stabilized after a 16-hour incubation period (55% and 67% of P-H2AX-positive cells at the dose corresponding to the antiproliferative EC_{50} and 2 μ mol/L, respectively). The presence of Ser-139-phosphorylated H2AX was also explored as an *in vivo* PD biomarker of F14512.

P-H2AX is a PD biomarker of F14512 in dogs

As a proof of concept of F14512-targeting tumors in dogs with naturally occurring lymphoma, we looked at P-H2AX induction in tumoral lymph node fine-needle aspirates. Four lymphoma-bearing dogs received a single F14512 i.v. injection at a low dose of 0.05 mg/kg, and serial fine-needle aspirates were performed. A

P-H2AX induction was observed by flow cytometry as early as 2 hours (not shown) after the end of the F14512 infusion and increased at 4 hours (Fig. 1). P-H2AX induction was heterogeneous among the 4 patients but an increase was observed in all dogs. These PD data further supported the clinical evaluation and dose-escalation trial of F14512 in lymphoma-bearing dogs. These 4 dogs were subsequently included in the first cohort of the trial.

Epidemiologic characteristics and staging of dogs with spontaneous lymphoma

Twenty-three dogs with naturally occurring lymphomas were enrolled in the dose-escalation trial consisting of three cycles of F14512 i.v. injection between November 2013 and March 2014. The epidemiologic characteristics of these patients are summarized in Table 1. There were 14 female and 9 male dogs. The mean age of all 23 dogs was 8.0 ± 2.6 years. The mean weight of all dogs was 29.5 ± 17 kg. Seventeen different canine breeds known as being predisposed to lymphoma were represented (32). The majority of cases (15/23) were classified as DLBCLs, 12 as centroblastic (DLBCL-CB) and 3 as immunoblastic (DLBCL-IB). Three other B-cell lymphoma cases were identified as late-stage marginal zone lymphomas in transition into DLBCL-IB. The other subtypes were: two peripheral T-cell lymphomas (2/23), including one pleomorphic small lymphoma and one pleomorphic mixed lymphoma (according to the updated Kiel Malignant Lymphoma Classification); and a T-cell lymphoblastic lymphoma (1/23). The lymphoma subtype could not be determined in two cases due to the lack of biopsy material, but the diagnosis of high-grade lymphoma was based on the cytologic examination. Two dogs had previously received long-term corticosteroid monotherapy before the study, with partial responses and rapid relapses. Four dogs had previously received various CHOP-based chemotherapy treatments with a complete response and relapse before inclusion.

Pharmacokinetics

A total of five cohorts were successively initiated, with an initial dosage of 0.050, 0.060, 0.070, 0.085, and 0.075 mg/kg (cohorts 1 to 5). Although the cohort size per dose level was small and the dose range explored was narrow, F14512 and F16490 plasma AUC increased overall with the dose level (Fig. 2A). Figure 2B and C represents plasma concentrations versus time of F14512 and its metabolite F16490 on day 1 for all dogs treated at 0.075 mg/kg (recommended dose). F14512 plasma concentrations were in the range of the IC_{50} value estimated in the Namalwa model (46 nmol/L = 29 ng/mL) for approximately 2 to 3 hours in most dogs. Interestingly, the patient with the lowest plasma concentration was the only one that did not have a clinical response to treatment at this dose level. Elsewhere, the AUC of the active metabolite F16490 and of F14512 was proportional and, on average, the F16490 AUC represented 23% of the F14512 AUC.

Pharmacodynamics

Lymph node tumor cells and blood cell numeration were monitored early during this clinical trial to determine whether F14512 therapy was associated with any biologic effect. Serial fine-needle aspirates were performed in tumoral lymph nodes in the hours following the first injection of F14512 to look for PD markers of F14512. The total cell number in these serial aspirates was evaluated and normalized to the aspirate volume. A rapid and

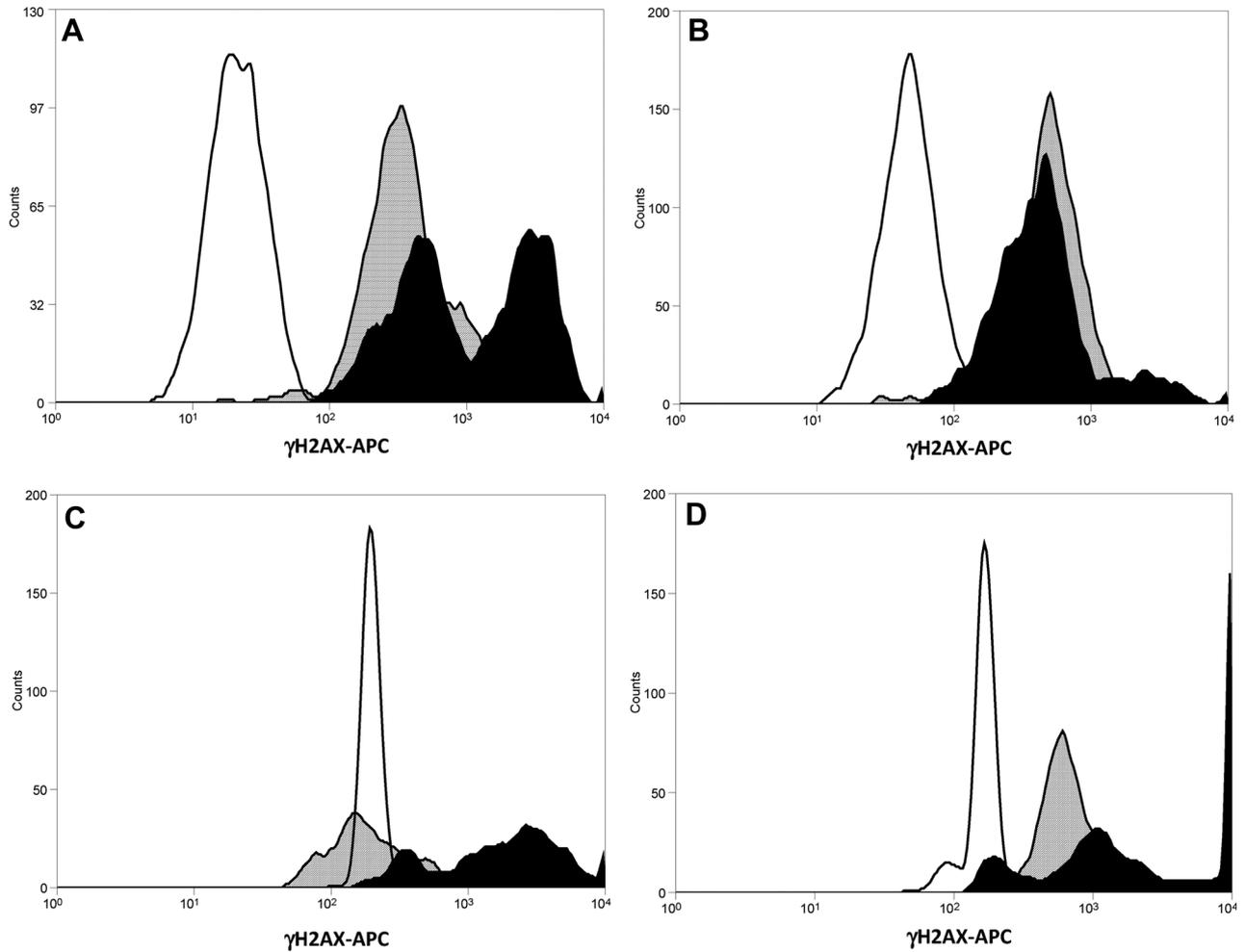


Figure 1.

P-H2AX is a PD biomarker of F14512 in dogs. *In vivo* analysis of P-H2AX staining in fine-needle aspirates of tumor lymph nodes from 4 dogs (A-D) treated with 0.05 mg/kg F14512. Isotype control (blank), before treatment (shaded) and 4 hours after the end of F14512 infusion (black).

dramatic decrease in the number of cells was observed (Fig. 3A) as early as 2 hours after the beginning of the F14512 infusion. By the end of the first cycle of the F14512 therapy, the number of cells was dramatically reduced. Such a potent effect on tumoral cells made the analysis of P-H2AX induction impossible in all patients. Indeed, F14512 induced a strong decrease in viable cells, generating a lot of necrotic cells and debris (Supplementary Fig. S3), and thus rendering any reliable P-H2AX evaluation using flow cytometry impossible. This significant decrease in total lymph node cell numbers was noticed at all dose levels (not shown) and was clearly a PD marker of the F14512 efficacy.

Blood cell counts could be both a marker of efficacy and toxicity. As expected, a decrease in the white blood cell count, including neutrophils, lymphocytes, and monocytes, was observed after each cycle of F14512 therapy, with a nadir on day nine. A dose–effect relationship was observed between the dose of F14512 and the number of neutrophils (Fig. 3B). This decrease was reversible and baseline levels were recovered by the beginning of the next cycle. The evaluation of the decrease in white blood cells as well as the duration of the decrease correlated with the dose of F14512 and allowed to determine DLTs.

Toxicities

All 23 dogs were evaluated for tolerance. Signs of toxicity included neutropenia, anemia, thrombocytopenia, and digestive disorders (diarrhea and vomiting). Toxicities are reported in Table 2. Gastrointestinal adverse events (diarrhea for 6 dogs and vomiting for 2 dogs) were mild in 6 dogs (grade 1) and moderate in 1 dog (grade 2). They were not dose related (no gastrointestinal toxicity observed with the highest dosage) and the side effects resolved rapidly with the use of symptomatic treatments. Severe adverse events (grade 3 and above) were limited to neutropenia, thrombocytopenia, and anemia, and were all reversible. Grade 4 neutropenia was observed at all dosages, but was short (less than 48 hours) and clinically well tolerated for the first three dose levels. Cohorts 1 and 2 were extended to confirm the short duration and reversibility of the neutropenia at these dose levels. Grade 4 neutropenia lasting more than 48 hours was observed in 2 dogs from cohort 4, and was considered as a DLT. A fifth cohort with an intermediate dosage (0.075 mg/kg) was, therefore, constituted and determined as the recommended dose. There were no treatment-related deaths, but 1 dog died due to the progress of the disease on day 27.

Table 1. Epidemiologic and clinical characteristics at diagnosis of the whole study population composed of 23 dogs with naïve and relapsing lymphoma

Epidemiologic characteristics	Population studied
Sex	
Male	39.1% (9/23)
Female	60.9% (14/23)
Age, y, mean ± SD (range)	8.0 ± 2.6 (4.0–15.0)
Body weight, kg, mean ± SD (range)	29.5 ± 17 (4.3–54.0)
Clinical characteristics	
Breed	
Rottweiler	13.0% (3/23)
Cavalier King Charles spaniel, Bernese mountain dog, Jack Russel terrier, Labrador Retriever	8.7% (2/23 each 4 breeds)
Yorkshire terrier, Cocker spaniel, West highland white terrier, Golden Retriever, Bull terrier, French Bouledogue, Dogue de Bordeaux, Weimaraner, Mastiff, Greyhound, German Shepherd, cross-breed	4.3% (1/23 each 12 breeds)
Clinical stage	
Stage 3	17.4% (4/23)
Stage 4	82.6% (19/23)
Clinical substage	
a	52.1% (12/23)
b	47.9% (11/23)
Hypercalcemia	8.7% (2/23)
Pretreatment	
Corticosteroid	8.7% (2/23)
Chemotherapy	17.4% (4/23)
Lymphoma subtype	
B cell	
Diffuse large B-cell lymphoma	65.2% (15/23)
Marginal zone lymphoma	13.0% (3/23)
T cell	
Peripheral T cell	8.7% (2/23)
T-cell lymphoblastic	4.3% (1/23)
Unclassified	8.7% (2/23)

Clinical outcome

Stable or progressive disease was observed in 2 dogs, both having been treated before entering the study (Table 3). One of these dogs, treated in cohort 1, died on day 27 with progressive disease, whereas the other dog, treated in cohort 5, died on day 128 (after showing a stable disease on day 45). Ten dogs achieved a complete regression, whereas 11 dogs experienced a partial remission (Table 3). Pretreatment and inclusion in one of the first three cohorts were associated with a tendency toward a lower chance of achieving a complete remission, although the difference was not statistically significant. Twenty dogs received additional treatments following F14512 (consisting of various chemotherapy protocols).

Discussion

Naturally occurring canine tumors represent a useful and valuable model for deciphering many aspects of human cancers (33). As part of the broader field of comparative oncology, translational drug development studies in dogs with spontaneous cancers have been used to define doses and schedules for therapeutic agents through rigorous PK–PD endpoints, often involving serial biopsies of tumor tissue and the collection of biologic materials before and after exposure to novel therapeutics (34, 35). Comparative oncology has been focusing on the study of homologies, differences, and translational relevance of various cancers, including lymphomas (36), osteosarcomas (37, 38), soft tissue sarcomas (39), urinary bladder cancer (40), mammary cancers (41), and others.

In this dose-escalating study, we have shown that the response rate (complete and partial responses) of F14512 in treated canine patients with lymphoma is 91%. Direct comparison of median

survival time or time to relapse with published data with other chemotherapy agents is difficult because of the heterogeneity of our population. Moreover, for obvious ethical reasons, some patients were treated with another type of chemotherapy after the F14512 study. Nevertheless, we can compare the clinical response rate with F14512 with the reported response rate with doxorubicin, which is commonly recognized as the most efficient single agent for the treatment of canine high-grade non-Hodgkin lymphoma. In a previous study (42), the response rate of dogs treated with doxorubicin as a first line agent was 74%, which can be compared with the result observed in our study (91%). Therefore, our results are promising and emphasize the potential of F14512, which could be investigated further either alone or in combination with other agents. Because the treatment schedule only included three cycles of F14512 injections, additional cycles could be considered in future studies to potentially increase clinical efficacy. As the main toxicity of F14512 is febrile neutropenia, a combination with conventional chemotherapy should be chosen rationally to avoid major toxicities. In human lymphomas, the CHOP-based chemotherapy is associated with anti-CD20 antibodies. It should be noted that some laboratories are now working on therapeutic canine anti-CD20 antibodies (43). Apart from rituximab, no other targeted therapy has emerged and been developed so far for DLBCL, and the canine patient as a cancer model may accelerate research. Ibrutinib, an irreversible BTK inhibitor, benefited from its evaluation in canine lymphomas (44) and is now proven to be efficacious in humans (45): The canine lymphoma model enabled the authors to demonstrate the full-target occupancy at various dosages, and to achieve some objective clinical responses (44). Second-generation BTK inhibitors are now being evaluated in dogs (46) in proof-of-concept studies along with their development pathway, whereas clinical

A

	Dose level (mg/kg)				
	0.05	0.06	0.07	0.075	0.085
<i>N</i>	5	3	3	8	4
F14512 AUClast (h*ng/mL)					
Min	38.7	52.7	62.5	36.2	73.7
Median	45.2	83.4	115	142	126
Max	73.8	102	261	224	152
F16490 AUClast (h*ng/mL)					
Min	8.89	27	21.4	13.9	27.7
Median	12.6	28.3	25.2	36.2	35.2
Max	20.5	29.9	38.5	42.7	40.6

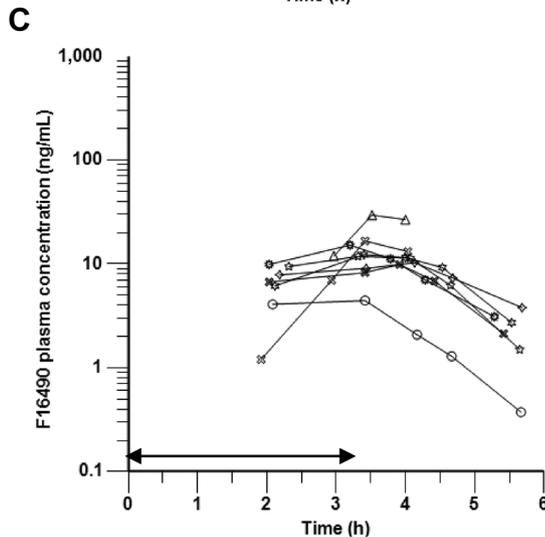
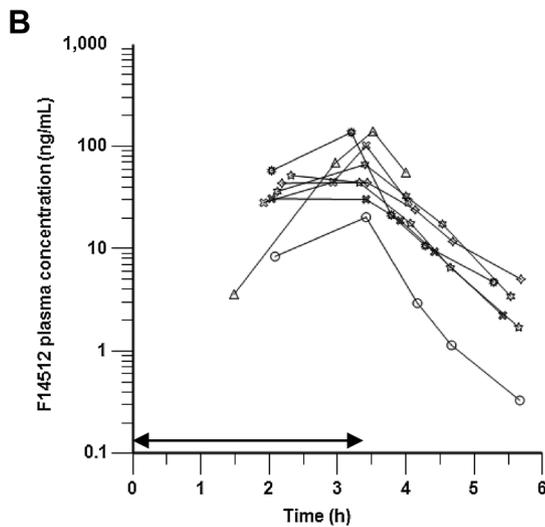


Figure 2. F14512 PK data in dogs. A, descriptive statistics of F14512 and F14512 main metabolite (F16490; AUClast by dose level). B and C, F14512 and F16490 plasma concentrations versus time for all dogs treated at the recommended dose level of 0.075 mg/kg, after the first infusion (cycle 1, day 1). F14512 infusions started at *t* = 0 hours and lasted between 3.25 and 3.63 hours (↔).

studies of combination of ibrutinib with polychemotherapy are ongoing in DLBCL in humans. Thus, we believe that a combination of F14512 with targeted therapies such as anti-CD20 mAb or BTK inhibitors should be considered in the future.

As a vectorized form of etoposide, F14512 was shown to be more potent than etoposide *in vitro* (4) and to be superior to etoposide in terms of efficacy and therapeutic window in xenograft mice models (8, 9). F14512 also revealed a convincing antitumor activity in naturally occurring lymphomas in dogs, with 22 of 23 patients displaying a clinical response (either stable disease, partial, or complete remission). These clinical results in dogs clearly appear to be superior to those described for etoposide (24). Etoposide was also shown to be poorly active in a retrospective study on 13 animals, with only 2 dogs displaying a

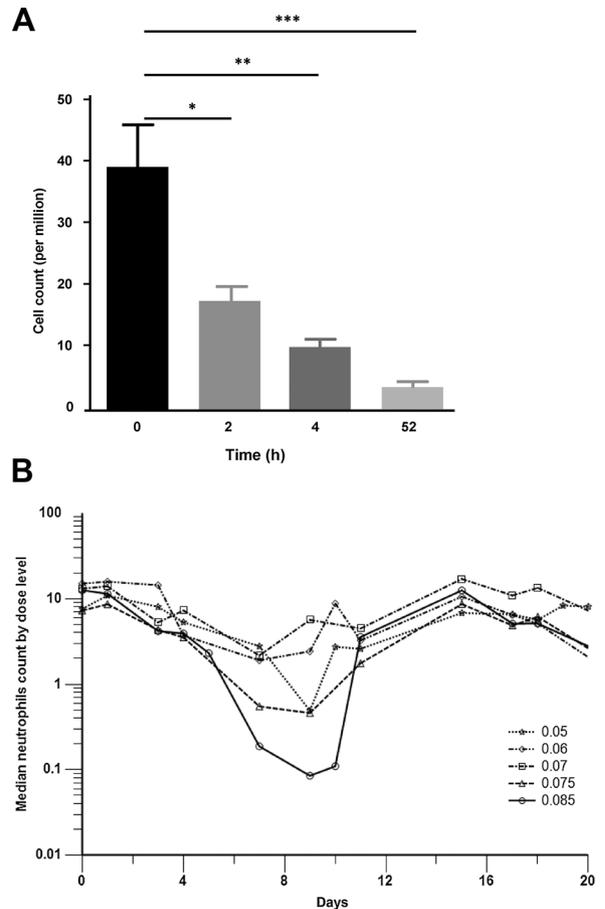


Figure 3. Tumoral and hematologic PD markers of F14512. A, F14512 induces a strong and early decrease of tumoral lymph node cell number in dogs. Fine-needle aspirates were performed in tumoral lymph nodes all along the first cycle of F14512 administration. F14512 infusions were initiated at *t* = 0 hours and lasted 3 hours at day 1 (0–3 hours), day 2 (24–27 hours), and day 3 (48–51 hours). The graph shows the total cell count per milliliter of fine-needle aspirates, from all dogs treated at all dose levels (results, mean ± SD; data were analyzed using ANOVA followed by PLSD Fisher *post hoc*; *, *P* < 0.05; **, *P* < 0.01; and ***, *P* < 0.001, *n* = 23 dogs). B, F14512 induces a dose-dependent decrease in circulating neutrophils in treated dogs. The graph shows the dose-effect relationship between the dose level of F14512 and the number of neutrophils (median absolute neutrophil count vs. time by dose level in mg/kg). F14512 was administered on days 1, 2, and 3.

Table 2. Toxicity observed in the whole study population composed of 23 dogs with naïve and relapsing lymphoma

Cohort (dose)	Number of dogs	Hematologic toxicity grade (number of dogs)				Gastrointestinal toxicity grade (number of dogs)	
		Hemoglobin	HCT	Platelet	Neutrophils	Diarrhea	Vomiting
Cohort 1 (0.05 mg/m ²)	6	Grade 2 (1)	Grade 2 (1)	Grade 3 (1)	Grade 4 (3) ^a	Grade 1 (1)	Grade 1 (1)
		Grade 1 (4)	Grade 1 (4)	Grade 2 (3)	Grade 3 (1)		
Cohort 2 (0.06 mg/m ²)	4	Grade 4 (1)	Grade 4 (1)	Grade 2 (2)	Grade 4 (2) ^a	Grade 1 (2)	Grade 1 (1)
		Grade 2 (1)	Grade 3 (1)	Grade 1 (1)	Grade 3 (1)		
		Grade 1 (1)	Grade 2 (1)				
Cohort 3 (0.07 mg/m ²)	3	Grade 3 (1)	Grade 3 (1)	Grade 2 (1)	Grade 4 (1) ^a	Grade 2 (1)	0
		Grade 1 (2)	Grade 1 (2)		Grade 3 (1)		
Cohort 4 (0.085 mg/m ²)	4	Grade 2 (1)	Grade 2 (2)	0	Grade 4 (2) ^b	0	0
		Grade 1 (3)	Grade 1 (2)		Grade 4 (1) ^a		
Cohort 5 (0.075 mg/m ²)	6	Grade 2 (3)	Grade 2 (4)	Grade 4 (1)	Grade 4 (1) ^b	Grade 1 (2)	0
		Grade 1 (3)	Grade 1 (2)	Grade 3 (1)	Grade 4 (2) ^a		
				Grade 2 (2)	Grade 3 (1)		

^aAsymptomatic neutropenia lasting less than 48 hours.

^bFebrile neutropenia lasting more than 48 hours.

clinical response. Moreover, as F14512 was shown to be a poor substrate of the efflux protein PgP (unpublished data), it would be of great interest to look for F14512 efficacy in relapsing and/or refractory chemoresistant dog lymphomas who have a poor prognosis (47). The canine multidrug resistance associated protein has been molecularly identified (48). In the present study, 4 dogs had a previous polychemotherapy course. Even if their PgP/Mdr status was not known, a clinical response was observed in 3 of them. Therefore, this F14512 phase I study warrants further evaluation of F14512 in relapsing and/or chemoresistant lymphoma cases. Because of the unequal distribution of dogs depending on the lymphoma subtype, it was not possible to see any relation between the clinical response and the lymphoma subtype.

In this study, we looked at F14512-induced DNA damages and demonstrated that P-H2AX signaling (49) could be used as a PD biomarker. We took the opportunity to design a preliminary study in 4 dogs treated with a single injection of low-dose F14512 and unraveled an early *in vivo* P-H2AX induction as a PD response. Unfortunately, we could not correlate P-H2AX induction with the F14512 AUC or the clinical response owing to the rapid onset of lymphoma cell death induced by F14512. We were not able to recover enough living cells from all treated dogs to perform a relevant flow-cytometry study of P-H2AX. We did not expect such a tumoral cell death (as early as a couple of hours after initiating F14512 infusion). A preferential and rapid uptake of F14512 by tumor cells, with an added cytotoxic effect brought by F16490 metabolite, may explain this strong tumoral cytotoxicity. We previously demonstrated the preferential uptake of F14512 by

tumor cells (4, 5), and as a consequence plasma levels of F14512 and F16490 may only partially reflect the concentrations reached in tumors. F16490 alone displays cytotoxic activity with an EC₅₀ of 74 nmol/L in the Namalwa cell line. This rapid cell death induction was observed in all dogs, even in the 2 dogs that did not display a clinical response later. This latter observation confirms the strong cytotoxicity of F14512 in dog lymphoma and supports further evaluation either in combination and/or with the addition of other cycles of treatment to reinforce and extend its efficacy. Careful monitoring of patients may be required in human clinical trials for the early detection of tumor lysis syndrome that may occur.

An interesting point about the study we performed on dogs is that there is an ongoing clinical assessment of F14512 in humans, so we are able to compare and translate the clinical data and findings from both species, even if the clinical tumor indications are not the same. The first-in-man multicenter phase I trial on F14512 as a single drug was conducted in adult patients with relapsed or refractory AML (12, 50). Patients received a daily i.v. infusion for 5 consecutive days every 2 to 6 weeks depending on the leukemia response as well as the recovery of sufficient hematopoiesis and the resolution of toxicities. The main toxicity was myelosuppression, which was dose dependent and reversible. The MTD reached was 44 mg/m² and the recommended dose was determined to be 39 mg/m². Antileukemic activity was observed at different dose levels with 10% complete responses, 8% complete responses with incomplete recovery and 3 patients who experienced hematologic improvements. As with humans, we

Table 3. Clinical response of the whole study population composed of 23 dogs with naïve and relapsing lymphoma

Population	Clinical response at day 45			
	Progressive disease	Stable disease	Partial response	Complete response
Whole population	4.4% (1/23)	4.4% (1/23)	47.8% (11/23)	43.5% (10/23)
Pretreatment				
Yes (n = 6)	16.7% (1/6)	16.7% (1/6)	66.6% (4/6)	0
No (n = 17)	0	0	58.8% (10/17)	41.2% (7/17)
Cohort				
Cohort 1: 0.050 mg/kg (n = 6)	16.7% (1/6)	0	50.0% (3/6)	33.3% (2/6)
Cohort 2: 0.060 mg/kg (n = 4)	0	0	75.0% (3/4)	25.0% (1/4)
Cohort 3: 0.070 mg/kg (n = 3)	0	0	66.6% (2/3)	33.3% (1/3)
Cohort 4: 0.085 mg/kg (n = 4)	0	0	50.0% (2/4)	50.0% (2/4)
Cohort 5: 0.075 mg/kg (n = 6)	0	16.7% (1/6)	16.7% (1/6)	66.6% (4/6)

also observed clinical responses in dogs at all dose levels, but relapses were more frequently noticed at the first dose levels (even if not statistically significant). When we looked at PD markers of F14512 in dogs, the tumor lymph node cell counts and the circulating blood cells counts (Fig. 3) revealed a favorable therapeutic window: A strong decrease of tumor cells was observed at all doses whereas the hematologic adverse events were increased only at higher doses. Interestingly, the tolerance profile was very similar between humans and dogs, with the hematotoxicity being the major adverse event observed. A phase II trial of F14512 in combination with cytosine arabinoside is currently ongoing in patients with AML.

In conclusion, our results provide a strong evidence of the clinical efficacy of the new vectorized drug F14512 in a pet dog model of lymphoma. The translational value of canine lymphoma warrants further studies of F14512 and strongly supports its clinical development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

The authors thank Marie Bosredon for her kind assistance in the F14512 batch management and the flow core facility of BICeL-IFR114 for the technical assistance.

Grant Support

This work was supported by grant from BPI-France, through the CaninCa project.

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Received December 8, 2014; revised June 13, 2015; accepted July 4, 2015; published OnlineFirst July 13, 2015.

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