[CANCER RESEARCH 50, 6823-6826, November 1, 1990]

Pharmacologically Directed Design of the Dose Rate and Schedule of 2',2'-Difluorodeoxycytidine (Gemcitabine) Administration in Leukemia[1]

Ralf Grunewald, Hagop Kantarjian, Michael J. Keating, James Abbruzzese, Peter Tarassoff, and William Plunkett[2]

Departments of Medical Oncology [R. G., J. A., W. P] and Hematology [H. K., M. J. K.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, and Lilly Research Laboratories [P. T.], Indianapolis, Indiana 46285

ABSTRACT

The objective of this study was to determine the dose rate of 2',2'-difluorodeoxycytidine (dFdC) that maximizes the accumulation of the active 5'-triphosphate (dFdCTP) in circulating leukemia cells during therapy. The investigational approach was to evaluate the relationship between plasma dFdC and the accumulation of dFdCTP by circulating leukemia cells during infusion of different dFdC dose rates in the same individuals. Four patients with relapsed leukemia were treated weekly with two or three consecutive infusions of 800 mg/m², the first administered over 1 h, the second over 2 h, and the third over 3 h. Two patients, one with acute myelogenous leukemia and one with acute lymphocytic leukemia, received all three infusions, but thrombocytopenia prohibited infusion of the third dose to two patients with chronic lymphocytic leukemia. The average steady-state plasma dFdC levels, achieved within 15 min after the infusion began, were 43.8 μM during infusion of 800 mg/m²/h, 9.4 μM during infusion of 400 mg/m²/h, and 5.6 μM at 267 mg/m²/h. The median area under the concentration times time curve of dFdCTP in leukemia cells during infusion was increased 2.3- and 5.1-fold for the 2- and 3-h infusions, respectively. *In vitro* incubations of leukemia cells from the four patients with 2.5-100 μM dFdC for 1 h showed that the maximum cellular accumulation of dFdCTP was produced by 15-20 μM dFdC. We conclude that a dose rate of >400 mg/m²/h was required to achieve plasma dFdC levels that supported the maximum rate of dFdCTP accumulation in leukemia cells.

INTRODUCTION

dFdC[3] is a new nucleoside analogue antimetabolite that differs from deoxycytidine by the presence of geminal fluorines at the 2' position of the carbohydrate moiety (1). The broad spectum of activity exhibited by dFdC against murine leukemias and solid tumors, which was more impressive than that of ara-C (2), stimulated interest in a clinical trial. Cytotoxicity is associated with a specific inhibition of DNA synthesis, which is dependent upon the cellular accumulation of dFdC nucleotides (3, 4). Deoxycytidine kinase is required for phosphorylation of dFdC to the monophosphate; enzymes responsible for the subsequent phosphorylation to the di- and triphosphate have not been identified (3, 4). dFdCTP is the major nucleotide derivative of dFdC; its cellular concentration appears to be in a constant ratio with the mono- and diphosphates (3). dFdC 5'-diphosphate inhibits ribonucleotide reductase and decreases cellular deoxynucleotide pools in a concentration-dependent manner (5-7). The decrease in cellular dCTP may lead to self-potentiation of dFdC metabolism and action by several mechanisms. First, feedback inhibition of deoxycytidine kinase by dCTP (8, 9) is released, resulting in a higher activity of the enzyme which then can phosphorylate dFdC more rapidly (4, 5). Second, dFdCTP competes with dCTP for DNA polymerase and incorporation into DNA (10); lower cellular dCTP concentrations will favor utilization of the analogue. Finally, dFdC monophosphate is a substrate for dCMP deaminase; the velocity of this reaction is stimulated by dCTP (11). Reduction of dCTP pools may decrease the rate of dFdC monophosphate deamination, thus contributing to the prolonged retention of dFdCTP (3, 5).

Our phase I trial of dFdC in patients with solid tumors indicated that accumulation of dFdCTP by mononuclear cells is saturated by dose rates (225-350 mg/m²/30 min) that achieve 15-20 μM dFdC in plasma (12). Saturation kinetics had previously been demonstrated for the formation of ara-CTP in circulating leukemia cells during high-dose ara-C therapy (13, 14). These studies provided a pharmacological basis for "intermediate-dose ara-C," and clinical trials of the pharmacologically specified infusion rates demonstrated good antileukemia activity with reduced toxicity relative to high-dose regimens (15). Furthermore, strong correlations have been established between clinical response and the pharmacokinetics and pharmacodynamics of ara-CTP in leukemia cells (16-18). We therefore postulated that understanding the relationship of the dFdC concentration in plasma to the rate of dFdCTP accumulation by leukemia cells would be an important factor in protocol design.

The objectives of the present study were (*a*) to investigate the concentration of dFdC that saturates the accumulation of dFdCTP in leukemia cells *in vitro*, (*b*) to evaluate the ability of different dose rates to maximize dFdCTP accumulation in circulating leukemia cells, and (*c*) to determine the dose rate of dFdC that will achieve optimal dFdC concentrations in blood during therapy. This pharmacological information would be used in the design of a treatment protocol for patients with relapsed leukemia.

PATIENTS AND METHODS

Patients and Therapy. Four patients with relapsed leukemia were treated at weekly intervals with infusions of 800 mg/m² dFdC. Each patient was fully informed as to the nature of the investigations and provided consent to participate in the studies. To compare the rates of dFdCTP accumulation by leukemia cells in the presence of different steady-state dFdC concentrations in the plasma, the protocol was designed to infuse a total dose of 800 mg/m² over 1, 2, or 3 h, i.v., by pump on consecutive weeks (infusion rates: 800 mg/m²/h the first week, 400 mg/m²/h the second week, and 267 mg/m²/h the third week) for all four patients.

The patients' diagnoses were acute myelogenous leukemia (patient 1, a 47-year-old woman), acute lymphocytic leukemia (patient 2, a 43-year-old man), T-cell CLL (patient 3, a 68-year-old woman), and B-cell CLL (patient 4, a 54-year-old woman). Patients 1 and 2 received all three infusions, whereas thrombocytopenia prevented the infusion of

Received 4/18/90; accepted 7/30/90.

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[1] This work was supported in part by grant CA32839 from the National Cancer Institute, Department of Health and Human Services, and grant CH-130 from the American Cancer Society.

[2] To whom requests for reprints should be addressed, at Department of Medical Oncology, Box 52, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

[3] The abbreviations used are: dFdC, 2',2'-difluorodeoxycytidine, Gemcitabine; ara-C, 1-β-D-arabinofuranosylcytosine: ara-CTP, the 5'-triphosphate of ara-C; AUC, area under the concentration times time curve; dFdCTP, the 5'-triphosphate of dFdC; CLL, chronic lymphocytic leukemia; HPLC, high-performance liquid chromatography.

the third dose in the two patients with CLL. All patients experienced transient reductions in their WBC and blast counts, but no objective responses were observed.

Blood Samples. Venous blood samples (10 ml) were drawn through a catheter in the opposite arm from that used for dFdC infusion and were placed into heparinized tubes that contained 5 μmol of the cytidine deaminase inhibitor tetrahydrouridine. Samples were drawn immediately before each infusion, every 10 min during the 1-h infusion and every 15 min during the 2- and 3-h infusions. Samples also were drawn at 0, 5, 10, 15, 30, and 60 min after the end of each drug infusion. The tubes were immediately placed in an ice bath and the plasma was obtained by centrifugation (500 × *g* for 5 min at 4°C) and stored at −20°C for subsequent HPLC assay.

Plasma Pharmacology. A reverse-phase HPLC method was used to separate and quantitate dFdC in plasma. The chromatographic system (Waters Associates Inc., Milford, MA) consisted of two model 6000A pumps, a model 680 gradient controller, a model 730 data module, an automatic sample injector (WISP model 710B), and a model 481 UV light detector. The detection wavelength was 275 nm. A reverse-phase analytical column μBondapak C_{18} (0.39 x 30 cm, Waters Associates) was used. The mobile phase consisted of buffer A, 0.5 M ammonium acetate (pH 6.8), and buffer B, 50% methanol in deionized water. A linear gradient from 100% buffer A to 60% buffer B was run at a flow rate of 1.6 ml/min over 30 min. The lower limit of detection for dFdC was 0.05 nmol; the standard curve for dose response of detection was linear between 0.05 and 18.0 nmol dFdC. The coefficient of variation for the precision of quantitation was determined as <7% for dFdC. Calculation of the concentration of dFdC in plasma was based on the amount of drug detected in a 20-μl sample and was done using individual standard curves. Steady-state concentrations of dFdC in plasma were calculated as the mean ± SD of values obtained between 15 min after the start and the end of the infusion.

Cellular Pharmacology. After plasma was removed from the blood samples, the cell pellet was resuspended in 40 ml of phosphate-buffered saline (NaCl, 8.1 g/liter; Na_2HPO_4, 1.14 g/liter; KCl, 0.22 g/liter; and KH_2PO_4, 0.27 g/liter) and the suspension layered over 10 ml of Ficoll-Hypaque (specific gravity, 1.077 g/ml). After centrifugation at 500 × *g* at 4°C for 20 min, the buoyant leukemia cells were removed and were diluted to a final volume of 10 ml with phosphate-buffered saline. Duplicate analyses of the mean cell number and cell volume were made with a Coulter Counter model ZM equipped with a 100-channel particle size analyzer (model C-1000). Procedures for extracting nucleotides from the leukemia cells have been described (3, 14).

An anion-exchange HPLC method was used to separate and quantitate dFdCTP from circulating leukemia cells (3). The standard curve for the dose response of dFdCTP detection was linear between 0.02 and 2.0 nmol of dFdCTP (correlation coefficient, 0.999). The amount of dFdCTP was quantitated using a preprogramed external reference compound kindly provided by Dr. Alina Sen of this laboratory. The cellular concentration of dFdCTP was calculated by dividing the nmol value of dFdCTP by the number of cells from which the sample was extracted and by the mean cell volume of each sample. This calculation assumes that dFdCTP was uniformly distributed in total cell water.

***In Vitro* Incubation of Leukemia Cells with dFdC.** Blood samples (10 ml) were drawn from the four patients before they received dFdC. Leukemia cells were isolated by Ficoll-Hypaque centrifugation and incubated *in vitro* with [5-^{3}H]dFdC (Lilly Research Laboratories, Indianapolis, IN), diluted with nonradioactive dFdC. The final concentrations in the incubation samples were 2.5, 5, 10, 15, 20, 25, 30, 40, 50, and 100 μM dFdC. The final volume of each incubation sample was 2.5 ml; the medium for incubation was RPMI 1640 containing 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY) and the incubation time was 1 h. The cell number in each incubation sample was 5×10^6 for patients 1 and 2, 1×10^7 for patient 3, and 5×10^7 for patient 4. Depending on the total number of leukemia cells obtained, either duplicate or triplicate incubations were conducted at each concentration. The specific activity of [5-^{3}H]dFdC stocks were 1.83×10^7 dpm/μmol for 10- through 100-μM incubations and 1.8×10^8 dpm/μmol for 2.5- and 5-μM incubations.

After incubation at 37°C and 5% CO_2 for 1 h, 10 ml of phosphate-buffered saline (4°C) was added to each sample before centrifugation for 5 min at 500 × *g* at 4°C. The supernatant was discarded, and the cell pellet underwent nucleotide extraction as described previously (14). Cell extracts were analyzed by anion-exchange HPLC. Separation was achieved with two model 6000A pumps, a WISP (710B) automatic sample injector, a model 680 gradient controller, a model 730 data module, model 484 UV light detector (Waters Associates), and a radioactive flow detector FLO-ONE\beta series A-200 (Radiomatic Instruments & Chemical Co., Inc., Tampa, FL). An anion-exchange column Partisil-10 SAX was used with a concave gradient (curve 7) that changed from 50% buffer C (0.005 M ammonium phosphate, pH 2.8) and 50% buffer D (0.75 M ammonium phosphate, pH 3.7) to 100% buffer D after 25 min; HPLC continued on 100% buffer D another 6 min before returning to initial conditions. The flow rate was 1 ml/min.

RESULTS

Metabolism of dFdC by Leukemia Cells. The *in vitro* studies were conducted to determine the dFdC concentration that would saturate the ability of leukemia cells to accumulate dFdCTP. Leukemia cells from four patients were incubated with dFdC at concentrations of 2.5–100 μM for 1 h. The intracellular dFdCTP increased with dFdC concentrations between 2.5 and 15 μM (Fig. 1). Cells from patient 3 showed a further dFdCTP increase when incubated with 20 μM dFdC, but dFdC concentrations >20 μM did not augment dFdCTP accumulation in any cell sample.

The intracellular dFdCTP levels in the cells of three patients attained a plateau value which did not change with higher dFdC concentrations. However, the dFdCTP levels in cells from patient 4 declined with each dFdC concentration >15 μM. A dose-dependent decrease in cellular dFdCTP accumulation has been observed in K562 cells (4) and in human mononuclear cells from healthy donors (19). Similar apparent substrate inhibition has also been observed for ara-CTP accumulation (19, 20). The individual plateau levels of dFdCTP were 90 μM (patient 1), 120 μM (patient 2), and 130 μM (patient 3). The maximum dFdCTP concentration in patient 4 was 200 μM (at 15 μM dFdC). Thus, there was substantial heterogeneity among patients in the ability of leukemia cells to accumulate dFdCTP; however, maximum dFdCTP was achieved by 15–20 μM exogenous dFdC. These findings suggest a target range of plasma dFdC that should maximize dFdCTP accumulation.

Plasma dFdC Pharmacology. To determine the dFdC dose rate that produces the target dFdC level of 15–20 μM in blood during therapy, we infused 800 mg/m^2 of dFdC using three different dose rates in each patient. The dFdC plasma concentrations reached a steady-state level 15 min after the start of

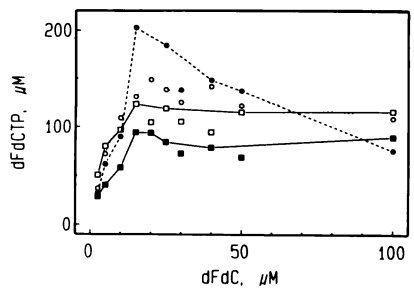

Fig. 1. Accumulation of dFdCTP by human leukemia cells *in vitro*. Before treatment, leukemia cells from four patients were isolated by Ficoll-Hypaque centrifugation and incubated for 1 h with the indicated concentrations of [5-^{3}H]dFdC. Cell extracts were analyzed for dFdCTP as described in "Patients and Methods." The values represent average results from either triplicate incubations (patient 1, ■; patient 3, ○; patient 4, ●) or duplicate incubations (patient 2, □).

infusion in all patients (data not shown). Table 1 lists the dFdC steady-state concentrations for each patient at the different dose rates. Dose rates of 400 and 267 mg/m²/h did not produce plasma dFdC levels >15 μM, which, according to the *in vitro* results, were necessary to maximize dFdCTP accumulation in these leukemia cells.

Cellular dFdCTP Pharmacology. dFdCTP accumulation by circulating leukemia cells from these patients increased linearly for at least 3 h (Fig. 2). The coefficient of determination for linearity of dFdCTP accumulation at each dose rate during the infusion had a range of 0.870–0.995. There was substantial heterogeneity in the dFdCTP accumulation rates among patients, the respective accumulation rates for the 1-, 2-, and 3-h infusions were 112, 90, and 82 μM/h in patient 1 (Fig. 2*A*); 104, 55, and 88 μM/h in patient 2 (Fig. 2*B*); 323 and 318 μM/h in patient 3 (Fig. 2*C*); and 146 and 82 μM/h in patient 4 (Fig. 2*D*). Thus, as expected, the highest dFdC dose rate was associated with a more rapid rate of dFdCTP accumulation in the cells of all patients, although proportionality between dFdC dose rate and the rate of dFdCTP accumulation was not always observed. This is consistent with the interpretation that, at the lower infusion rates, plasma dFdC levels were nearly sufficient to maximize nucleotide accumulation (*cf.* Fig. 2 and Table 1).

Table 2 lists the AUC values for dFdCTP throughout the infusions of the four patients. Again, there was heterogeneity in the absolute values among patients. Prolonging the duration of infusion of 800 mg/m² dFdC, however, clearly increased the accumulation of dFdCTP by leukemia cells. The median dFdCTP AUC for the 2-h infusion was 2.3 times that of the 1-h infusion, whereas the median dFdCTP AUC of the 3-h infusion was 5.1 times the 1-h infusion value.

DISCUSSION

In this study we sought to determine the dose rate of dFdC that achieves plasma levels maximizing the accumulation of dFdCTP in circulating leukemia cells during therapy. This is important because of the dose response between the intracellular exposure of the nucleotide and biological activity (3) and because dose rates that produce plasma levels of dFdC greater than those which can be metabolized by the leukemia cells may contribute to systemic toxicity.

Incubation of leukemia cells with dFdC *in vitro* demonstrated that dFdCTP accumulation was saturable; the maximum dFdCTP accumulation was achieved with 15–20 μM dFdC (Fig. 1). This finding is consistent with previous results in human K562 cells (4) and in mononuclear cells from healthy donors,[4] as well as with the results of the phase I dFdC study in patients with solid tumors (12). The importance of these relationships is highlighted by the fact that similar *in vitro* studies (20–22) predicted plasma ara-C concentrations that maximize ara-CTP accumulation (13, 14).

Plasma ara-C concentrations of 7–10 μM represent the lower limits for achieving maximum rates of ara-CTP accumulation in circulating leukemia cells (13, 14). Plasma levels in this range were achieved by ara-C infusion rates of 250 mg/m²/h. The dose rate that achieved dFdC plasma concentrations (20 μM) during our phase I study that saturated the accumulation of dFdCTP in mononuclear cells was 450–700 mg/m²/h. The proportionality between these dose rates and plasma levels reflects similarities in susceptibility of the two nucleosides to deamination (5). In contrast, the different concentrations associated with maximal triphosphate accumulation rates are consistent with findings in Chinese hamster ovary cells that indicate dFdC is a more effective permeant and a better substrate for deoxycytidine kinase than is ara-C (3). The role of self-potentiation of dFdCTP accumulation via an activation of deoxycytidine kinase due to a dFdCDP-induced decrease in dCTP pools (5, 11) remains to be elucidated fully, although the usefulness of dFdC as a modulator of the metabolism of other nucleosides phosphorylated by deoxycytidine kinase has been demonstrated (4).

The present study results suggest that dFdCTP accumulation in leukemia cells from 4 patients with different diagnoses was maximized at dFdC infusion rates that saturated triphosphate accumulation in mononuclear cells from a larger group of patients with solid tumor (12, 19). In future investigations, it should be possible to determine whether different leukemic morphologies are characterized by unique metabolic properties. Furthermore, it will be important to consider whether a differential ability between normal and leukemic cells to metabolize plasma dFdC could contribute to the therapeutic index of the drug.

The phase I study of dFdC demonstrated linearity of steady-state plasma dFdC increase with dFdC dose rates in 29 patients

Table 1 *Steady-state plasma concentrations of dFdC achieved by different dose rates*

Patients received infusions of 800 mg/m² of dFdC over 1, 2, and 3 h at weekly intervals, resulting in the indicated dose rates. The mean ± SD steady-state concentrations of dFdC in plasma, expressed as nmol/ml, were determined as described in "Patients and Methods."

	dFdC dose rate (mg/m²/h)		
Patient	800	400	267
1	25.7 ± 3.8	10.0 ± 1.7	5.1 ± 1.6
2	58.3 ± 23.1	9.9 ± 2.4	6.1 ± 1.2
3	62.4 ± 6.1	11.5 ± 1.0	
4	28.6 ± 7.9	6.0 ± 2.5	
Mean	43.8	9.4	5.6

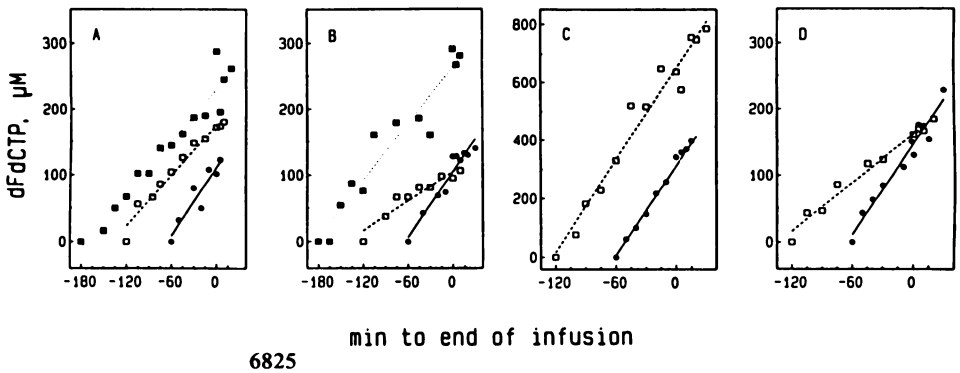

Fig. 2. Accumulation of dFdCTP by circulating leukemia cells during dFdC therapy. Four patients who had relapsed leukemia were treated with dFdC at weekly intervals. *A*, patient 1; *B*, patient 2; *C*, patient 3; *D*, patient 4. Infusions of 800 mg/m² dFdC were administered over 1 h (●), 2 h (□), or 3 h (■). Leukemia cells were isolated from blood samples drawn at the indicated times, and cellular dFdCTP concentrations were determined as described in "Patients and Methods."

Table 2 *dFdCTP AUC in leukemia cells during infusion of dFdC at different dose rates*

Patients received infusions of 800 mg/m² of dFdC over 1, 2, and 3 h at weekly intervals, resulting in the indicated dose rates. The AUC for dFdCTP, expressed in units of μM-h, were calculated for each patient from the data presented in Fig. 2.

Patient	dFdC dose rate (mg/m²/h)		
	800	400	267
1	66	212 (3.2)[a]	430 (6.5)
2	117	129 (1.1)	435 (3.5)
3	252	1012 (4.0)	
4	164	222 (1.4)	
Median	140	217 (2.3)	432 (5.1)

[a] Numbers in parentheses represent the AUC value relative to that for the 800 mg/m²/h dosage of dFdC.

infused over 30 min with doses between 53 and 1000 mg/m². In the present study, however, the steady-state levels did not increase in a linear mode with dose rates from 267 to 800 mg/m²/h (Table 1). It is likely that this can be attributed to the small sample number. The present results do indicate that a dose rate of 400 mg/m²/h did not produce the maximum rate of dFdCTP accumulation (Fig. 2); accordingly, a somewhat greater dose rate would be expected to achieve optimal dFdC steady-state levels in plasma. dFdC infusion at 800 mg/m²/h resulted in a mean of 43.8 μM dFdC in plasma (Table 1), which *in vitro* tests showed was more than required for maximum dFdCTP accumulation (Fig. 1). Based on these data, we have designed a phase I-II trial of dFdC in patients with relapsed leukemia in which dFdC is administered at an intermediate dose rate, 600 mg/m²/h. Starting with three weekly 2-h infusions, the protocol is designed to increase dose intensity by extending the infusion duration while maintaining the dose rate. A mean dFdC steady-state level of 28.7 ± 9.3 μM (SD) (range, 17–53 μM) has been observed in the 12 patients treated to date (23).

dFdC is eliminated from human plasma rapidly ($t_{½}$, 9 min), mainly because it is a good substrate for deoxycytidine deaminase (5, 12). However, the median terminal $t_{½}$ of dFdCTP elimination from mononuclear cells *in vivo* is 5 h [n = 23 (12)] and much longer in cell lines (3, 5). When the elements of dFdCTP metabolism by leukemia cells are considered, the rate of cellular elimination is likely to be a minor component relative to the overall rate of dFdCTP accumulation (Table 2). This indicates that the rate of synthesis is the governing factor in dFdCTP accumulation. After dFdCTP, dFdC is the major constituent of the cellular pool in cells incubated with >25 μM dFdC. This indicates that phosphorylation by deoxycytidine kinase is rate limiting in this pathway.

Evaluation of the postinfusion cellular pharmacokinetics of dFdCTP should receive high priority during subsequent leukemia trials. Heterogeneity in the elimination rates of dFdCTP may have prognostic value, as has been demonstrated for ara-CTP (16–18). This information may also impact on the dose schedule. For instance, if dFdCTP is eliminated entirely from leukemia cells in 1 or 2 days, more frequent infusions of dFdC should be considered. Thus, the cellular pharmacokinetics and pharmacodynamics of dFdCTP are likely to be useful parameters for optimizing infusions as well as foci for correlations with clinical response.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent technical assistance of Theresa Adams and Min Du and the editorial advice of Kathryn E. Baethge in the preparation of this manuscript.

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