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Original Research

Long-Term Effects of Sulfadiazine-Trimethoprim Medicated Diet on Cardiac Function, Hematology, and Weight Gain in Hsd:ICR (CD1) and Tac:SW Mice

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With the alarming increase in heart disease and heart failure, the need for appropriate and ethical animal models of cardiac dysfunction continues to grow. Currently, many animal models of cardiomyopathy require either invasive procedures or genetic manipulation, both of which require extensive expertise, time, and cost. Serendipitous findings at our institution revealed a possible correlation between sulfadiazine-trimethoprim (SDZ-TMP) medicated diet and the development of cardiomyopathy in IcrTac:ICR mice. We hypothesized that mice fed SDZ-TMP medicated diet continuously for 3 to 6 mo would develop cardiomyocyte degeneration and fibrosis, eventually leading to dilated cardiomyopathy. A total of 44 mice (22 Hsd:ICR (CD1) and 22 Tac:SW) were enrolled in the study. Half of these 44 mice were fed standard rodent diet and the other half were fed SDZ-TMP medicated diet. Baseline samples, including weights, CBCs, select biochemistry parameters, and echocardiography were performed prior to the start of either diet. Weights were obtained monthly and all other parameters were measured at least once during the study, and again at its conclusion. After 42 wk, mice were euthanized, and heart, lung and bone marrow tissue were submitted for histopathologic evaluation. Histologically, hearts were scored for the degree of degeneration, fibrosis, inflammation, and vacuolation. The data showed that SDZ-TMP did not have a significant effect on cardiac function, RBC parameters, biochemistry parameters (ALT, AST, calcium, magnesium, creatine kinase, and creatinine), hematopoiesis, or histologic heart scores. In addition, mice fed the SDZ-TMP medicated diet gained less weight over time. In summary, we were unable to reproduce the previous findings and thus could not use this approach to develop a novel model of cardiomyopathy. However, these results indicate that SDZ-TMP medicated diet containing 1,365 ppm of SDZ and 275 ppm of TMP does not appear to have long-term detrimental effects in mice.

Abbreviations: EF, Ejection fraction; FS, Fractional shortening; ICR, Hsd:ICR (CD1); LV, Left ventricular; LVIDd and LVIDs, Left ventricular internal diameter in diastole and systole; SDZ-TMP, Sulfadiazine-trimethoprim; SW, Tac:SW

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Heart disease is currently the leading cause of death in the United States. The American Heart Association estimates that the prevalence of heart failure will increase to 46% by 2030,[4] and the cost associated with heart disease is expected to rise from $55 billion in 2016 to $1.2 trillion (US dollars) by 2030.[4] The existing animal models of heart disease have enhanced our knowledge of its pathophysiology and has led to the development of numerous therapies.[9,30,47,54] However, many animal models of cardiomyopathy require invasive procedures such as aortic constriction,[16,58] left anterior descending artery ligation,[15,31,52] cryogenic injury,[27,57] or renal ischemia[68] to induce the cardiac changes associated with heart failure. The chemotherapeutic agent doxorubicin has also been used to induce heart failure in mice,[66,69] but a major disadvantage is the risk it poses to workers as a hazardous agent. Genetically engineered mice can replace invasive surgical models of cardiac disease,[14] but they also have significant limitations including cost, time, and failure to express the anticipated phenotype.[3,38,39] Despite advancements in cardiac research, reproducible and economical animal models of heart disease are still needed to further our understanding and explore novel therapeutic solutions.

Researchers at the University of Chicago reported that IcrTac:ICR sentinel mice that were accidently fed sulfadiazine trimethoprim (SDZ-TMP) medicated diet for 3 to 6 mo developed cardiomyopathy and subsequently cardiac dysfunction.[48] Prior to the onset of cardiomyopathy, these animals showed no signs of clinical disease.[48] SDZ-TMP is a potentiated sulfonamide, and the synergistic activity of sulfadiazine and trimethoprim allows it to not only inhibit folic acid metabolism in bacteria but also in some protozoa and fungi.[25] Potentiated sulfonamides are commonly administered to immunocompromised mice to prevent or treat opportunistic infections such as *Pneumocystis murina*.[20] However, SDZ-TMP may have contributed to the development of cardiomyopathy in these mice,[48]

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as potentiated sulfonamides are known to have adverse side effects in other animals, such as keratitis sicca, hypothyroidism, anemia, leukopenia, and hypersensitivity reactions.[50]

Based on the previous findings,[48] the goal of this study was to develop a cost-effective, reproducible, and noninvasive animal model of dilated cardiomyopathy. The prior data suggested that this model would have a slow and progressive onset, mimicking many aspects of heart disease and heart failure in humans.[48] We hypothesized that mice fed SDZ-TMP medicated diet continuously for 3 to 6 mo would develop cardiomyocyte degeneration and fibrosis, eventually progressing to dilated cardiomyopathy. Initial findings were discovered in the outbred stock IcrTac:ICR, but at the time of the present study, live production of this stock had ceased. Therefore, we elected to investigate our hypothesis in 2 readily available outbred stocks. We first attempted to monitor the progression of cardiomyopathy over time via echocardiography in Hsd:ICR (CD1) and Tac:SW mice fed SDZ-TMP medicated diet. In addition to capturing this progression, other objectives of this study were to investigate the long-term effects of SDZ-TMP medicated diet on other health parameters, including CBCs, a subset of biochemistry parameters to assess kidney and liver function, and weight gain. Thus, our goals were to both create an improved animal model of cardiomyopathy and enhance our understanding of prolonged prophylactic antibiotic use and its potential effects on biomedical research models and results.

Materials and Methods

Animals and colony health monitoring. Twenty-two Hsd:ICR (CD1) mice (age, 4 wk; 11 male; 11 female) (Envigo, Indianapolis, IN) and 22 Tac:SW mice (age, 4 wk; 11 male; 11 female) (Taconic, Germantown, NY) were used. On arrival, they were group housed in either pairs or trios, according to sex, and maintained in solid-bottom polysulfone IVC (19.69 × 30.48 × 16.51 cm; Jag 75 Micro-Barrier IVC, Allentown Caging). All cages, bedding, and enrichment were autoclaved prior to use. Each cage contained 1/4 in. corncob bedding (Teklad 7097, Envigo, Indianapolis, IN), a cotton square (Ancare, Bellmore, NY) and approximately 4 g of Enviro-dri (Shepherd Specialty Papers, Watertown, TN) as enrichment. All mice were fed irradiated standard rodent diet (Teklad 2918, Envigo, Indianapolis, IN), and provided with ad lib acidified water on arrival, prior to the start of any experimental manipulation. Animal rooms were maintained on a 12:12 h light:dark cycle with humidity ranging from 30% to 70% and temperatures ranging from 68 to 76 °F (20.0 to 24.4 °C) in compliance with the *Guide for the Care and Use of Laboratory Animals*.[33] Animals were checked daily by the animal care staff to check their health status and the availability of appropriate food, water, and cage conditions.

Routine colony health monitoring was performed quarterly via PCR testing using Sentinel EAD Collection Media (Allentown, Allentown, NJ), which was placed in the exhaust plenum of every IVC rack. Animals were considered free of the following viral, bacterial, and parasitic agents: mouse hepatitis virus, Sendai virus, pneumonia virus of mice, mouse parvovirus, minute virus of mice, Theiler murine encephalomyelitis virus, reovirus type 3, mouse rotavirus, ectromelia virus, lymphocytic choriomeningitis virus, mouse cytomegalovirus, mouse adenovirus 1 and 2, hantavirus, *Mycoplasma pulmonis*, *Salmonella* spp., *Citrobacter rodentium*, *Clostridium piliforme*, *Streptobacillus moniliformis*, *Filobacterium rodentium*, *Corynebacterium kutscheri*, pinworms (*Syphacia obvelata* and *Aspicularis tetraptera*), fur mites (*Myobia musculi*, *Myocoptes musculinus*, and *Radfordia affinis*), and *Giardia* spp. Organisms known to be endemic at the University of Chicago are *Helicobacter* spp., *Rodentibacter pneumotropicus* and *R. heylii* (previously *Pasteurella pneumotropica*), and mouse norovirus with the exception of a few designated rooms. All animal care and use were conducted in accordance with federal polices and guidelines and were approved by the University of Chicago's IACUC. The University of Chicago has a PHS assurance with OLAW, is a USDA registered research facility, and AAALAC International has accredited the animal care program since 2002.

Experimental design. After 1 wk of acclimation, animals were ear punched for identification purposes, and baseline data including weights, echocardiograms, and blood samples for CBC and biochemistry were obtained. The animals were randomly assigned to either irradiated standard rodent diet (Teklad 2918, Envigo, Indianapolis, IN) or irradiated SDZ-TMP medicated diet, containing 1,365 ppm of SDZ and 275 ppm of TMP (Teklad TD. 06596, Envigo, Indianapolis, IN). For both outbred stocks, 11 males and 12 females were assigned to SDZ-TMP medicated diet and 11 males and 10 females were assigned to standard rodent diet.

Cardiac function. For transthoracic echocardiogram assessment, mice were anesthetized with 1% to 3% isoflurane (Henry Schein Animal Health, Dublin, OH) as described.[23,55] Temperature was maintained at 37 °C using a heated EKG platform (Visual Sonics Vevo Integrated Rail System III, Toronto, Ontario) and heart rate monitored and maintained at or above 400 to 450 bpm. The fur on the ventral thorax was shaved with clippers, and any remaining hair follicles were removed using a topical depilatory agent. Two-dimensional images were recorded in parasternal long- and short-axis projections, with guided M-mode recordings at the midventricular level in both views using a 30-MHz high frequency transducer (Vevo 770 Fujifilm Visual Sonics, Toronto, ON, Canada). Left ventricular (LV) cavity size and percent fractional shortening were determined in at least 3 beats from each projection and then averaged. M-mode measurements were used to determine LV chamber dimensions and percent LV fractional shortening, which was calculated as ([LVIDd – LVIDs]/LVIDd), where LVIDd and LVIDs are LV internal diameter in diastole and systole, respectively). Echocardiograms were repeated at 12 to 14, 24 to 26, and 36 to 38 wks after the diets were implemented.

Clinical pathology. For biochemistries, animals were anesthetized with 2% to 4% isoflurane (Henry Schein Animal Health, Dublin, OH) and 200 uL of blood was obtained via the retroorbital sinus and placed into serum separator tubes. The collected samples were centrifuged and serum was pipetted into microcentrifuge tubes and frozen at -20 °C until analysis. One week after chemistry samples were collected, approximately 50 uL of blood was collected from the submandibular vein, using either a 4mm or 5mm lancet (MEDIpoint, Mineola, NY) in conscious mice for CBCs. Frozen serum and whole blood were sent to Comparative Clinical Pathology Services, (Columbia, MO) for analysis. Chemistries were performed on a Beckman Coulter AU680 Automated Chemistry Analyzer (Beckman-Coulter, Brea, CA), and CBCs were performed on a Procyte Dx Hematology Analyzer (Idexx Bioresearch, Colombia, MO). Blood sampling was repeated at 14 to 16 wk and 40 to 42 wk after the diets were implemented.

Body weight. Animals were weighed every 4 wk after beginning the 2 diets. Mice were removed from their cages, placed in a plastic dish on a scale (Ohaus CS 200, Parsippany, NJ), and weighed to the nearest 0.1 gm.

Gross necropsy. Mice displaying clinical signs of illness, such as lethargy, weight loss, ruffled fur, and/or tachypnea were

euthanized by CO_2 asphyxiation using a gas displacement rate of 10% to 30% of the chamber volume/minute.[37] Remaining mice were euthanized at the conclusion of the study (42 wk after diet implementation) via the same method. All organs were assessed for obvious signs of gross pathology. Any organs that showed discoloration, enlargement, or tumor growth were submitted for histopathology.

Histopathology. Lungs were inflated with 1 mL of 10% formalin (Thermo Fischer Scientific, Pittsburg, PA) prior to collection. The heart, lungs, and femurs were collected from each animal, fixed in 10% formalin, and were submitted for histopathologic evaluation to Idexx Bioresearch (Colombia, MO). After fixation, trimmed tissues were processed for paraffin embedding, sectioned, and stained with hematoxylin and eosin or Masson trichome. Microscopic evaluation of all slides was conducted by the same board-certified veterinary pathologist, who was blinded to sample identity. Changes in all tissues submitted were described. Heart sections were evaluated and scored for severity of changes associated with degeneration, fibrosis, inflammation, and vacuoles (Table 1). Degeneration was scored in 4 different areas: right ventricle (RV), interventricular septum (IVS), left ventricle (LV), and atrioventricular (AV) junction. Slides stained with Masson trichome stain were also scored for fibrosis in those same areas except for the AV junction. Inflammation was scored as either negative or present if mononuclear cells and/or fibroblasts were noted in any areas of degeneration, and vacuoles were scored based on their association within normal or degenerative cardiomyocytes. All other organs, including kidneys, liver, spleen, GI, skin, and tibia were collected, fixed, and stored for future analysis.

Statistical analysis. The data were inspected for stock differences, which were found only for heart scores. Therefore, data from both outbred stocks were combined for analysis of diet, time, and sex effects. Because of the higher variability in heart degeneration and fibrosis in the SW mice, the 2 stocks were analyzed separately for this parameter. For heart scores, a summed score was calculated over the ordinal or dichotomous component measures (degeneration, fibrosis, inflammation, and vacuoles) and compared between the SDZ-TMP medicated feed and standard groups using a Wilcoxon rank-sum test. For the remaining longitudinal variables, 3 analyses were performed. First, a summary measure equal to the average of the postbaseline values minus the baseline value was derived for each animal (δ 1) and compared between the antibiotic and standard groups using a 2-sample *t* test. Second, the change from the baseline value to the final time point value was calculated (δ 2) and the change was compared between groups using 2-sample *t* tests. For the third analysis, a mixed effects regression model was fit,[24] including sex, time, and a treatment-by-time interaction as fixed effects with animal as a random intercept. The main effect of interest was the treatment-by-time interaction, which was evaluated as the difference in slopes between the 2 treatment groups. Outliers from animals that died prior to completion of the experiment were excluded (one value each for WBC, ALT, AST, lymphocytes, monocytes, and neutrophils). Results were expressed as mean ± SD, and differences were considered statistically significant at a *P* value of less than 0.05.

Results

Animals. A total of 44 mice were enrolled in the study and a total of 39 mice completed the study. With regard to the other 5 mice, 3 died or were euthanized due to lymphoma, one died from a pheochromocytoma, and one died after the first submandibular blood collection. Table 2 describes the stock, sex, diet, and age of death for these animals. For the 39 mice (age, approximately 1 y) that completed the study, only one animal (SW-20) showed reduced cardiac function via echocardiography at the 36 to 38-wk time point. SW-20 was a female from the SDZ-TMP cohort. This animal was immediately euthanized after imaging due to cardiac decompensation, (see sections below for further details). The remaining animals were euthanized 42 wk after they began their respective diets.

Cardiac function. The results for FS, EF, and LVIDd are shown in Table 3 and Figure 1. Differences in the mean change comparing baseline to the average of all postbaseline values (δ 1) and differences in the mean change from baseline to the final time point (δ 2) were not significant for FS, EF, or LVIDd between the 2 diet groups (Figure 2 and Table 4). In addition, the mixed-effect regression model showed no sex, time, or diet effects for fractional shortening or ejection fraction. However, regression analysis did reveal an increase in the LVIDd over time in all mice ($P < 0.001$) that was not different based on diet ($P = 0.109$). At the 36 to 38 wk time point, SW-20 had a FS of 14% and an EF of 31%. Both had decreased substantially, as the means of the previous measurements from this mouse were 28% (FS) and 55% (EF).

Clinical pathology. The results for CBC and biochemistry parameters are listed in Tables 5, 6, and 7. No significant difference were found in RBCs, hemoglobin, HCT, neutrophils, eosinophils, or basophils between the 2 diet groups. Hemoglobin and HCT fell significantly over time in both groups ($P < 0.001$), but the means for both diet groups remained within the reference range[18] at all time points. No significant differences were noted for RBC indices (MCV, MCH, MCHC) between the 2 diet groups, but both groups showed a significant decrease in MCV and MCH over time ($P < 0.01$). The mixed-effect regression model showed a significant increase in reticulocytes in both diet groups ($P < 0.001$); this increase was more pronounced in the standard diet group ($P < 0.004$). In addition, a significant decrease in platelets was found in the SDZ-TMP diet group but not in the standard diet group ($P < 0.02$). The average means of the 2 postbaseline WBC values were significantly higher, relative to baseline values, in the standard diet group (δ 1; $P = 0.01$). No significant change was observed in either group when comparing the final values to baseline values (δ 2). The mixed-effect regression model showed a negative slope in the SDZ-TMP diet, signifying a significant decline in WBC over time ($P < 0.01$), as compared with the standard group. Similar changes were also seen in the lymphocyte count ($P < 0.01$). Moreover, monocyte numbers increased in both diet groups, but the increase over time was greater in mice fed the standard diet ($P < 0.01$).

Diet and time did not have a significant effect on ALT, AST, or calcium. Over time, creatinine and creatine kinase fell significantly in both groups ($P < 0.02$), and magnesium had a slight increase in both groups ($P < 0.031$). However, the mean levels of creatinine and magnesium for both diet groups remained within the reference range at all time points.[53] BUN decreased significantly in the SDZ-TMP medicated group and increased significantly in the standard diet group ($P < 0.0388$). This significance was nominal and only observed while comparing the means of δ 1. Furthermore, the means for both diet groups were within the reference range at all time points.[53]

Body weight. Mice were weighed monthly; however, only the mean body weight from the same 4 time points as echocardiograms are shown in Table 8. A significant weight gain occurred over time in both diet groups ($P < 0.01$). By the end of the 36 wk, mice fed standard diet had gained approximately 12.2 grams,

Table 1. Interpretation of heart scores.

Score	Degeneration	Fibrosis	Inflammation	Vacuoles
0	None	None	Negative	Within normal cardiomyocytes
1	Minimal	Minimal	Present	Within degenerative cardiomyocytes
2	Mild	Mild	N/A	N/A
3	Moderate	Moderate	N/A	N/A
4	Marked	Marked	N/A	N/A

Each area of the heart evaluated, that is RV, IVS, LV, and AV junction obtained its own score for degeneration and fibrosis (excluding AV junction). Inflammation and vacuoles were only scored once based on their presence, meaning the maximum score per mouse was 30.

Table 2. List of mice that died or were euthanized prior to the end of the study.

Stock	Sex	Diet	Age of death	Cause of death
ICR	Male	Standard	5 wk	Suspected hemorrhage
ICR	Male	SDZ-TMP	5 mo	Lymphoma
SW	Male	SDZ-TMP	5 mo	Lymphoma
SW	Female	Standard	9 mo	Lymphoma
ICR	Male	Standard	10 mo	Pheochromocytoma

No evidence of decreased cardiac function was assessed via echocardiography and no significant heart lesions were present on histology in these animals.

Table 3. Mean left ventricular function expressed as mean ± 1 SD.

	Baseline	12–14 wk	24–26 wk	36–38 wk
Standard diet fractional shortening (%)	29.11 ± 2.77	29.19 ± 2.27	29.40 ± 1.79	30.29 ± 1.25
SDZ-TMP diet fractional shortening (%)	29.65 ± 2.33	28.71 ± 1.53	29.32 ± 1.29	28.96 ± 4.55*
Standard diet ejection fraction (%)	56.70 ± 4.20	56.59 ± 3.47	56.78 ± 2.81	58.22 ± 1.91
SDZ-TMP diet ejection fraction (%)	57.68 ± 3.84	56.05 ± 2.68	56.85 ± 1.93	56.33 ± 7.24*
Standard diet LVIDd (mm)	3.54 ± 0.32[a]	3.91 ± 0.45[a]	4.06 ± 0.36[a]	3.93 ± 0.41[a]
SDZ TMP diet LVIDd (mm)	3.54 ± 0.30[b]	3.83 ± 0.35[b]	3.84 ± 0.40[b]	3.84 ± 0.32[b]

Parameters were not significantly different between diet groups, however LVIDd did increase over time in both groups ($P < 0.001$).[a, b] At the 36-38 wk time point, SW-20 had decreased cardiac function, therefore the higher standard deviation noted during this time period.*

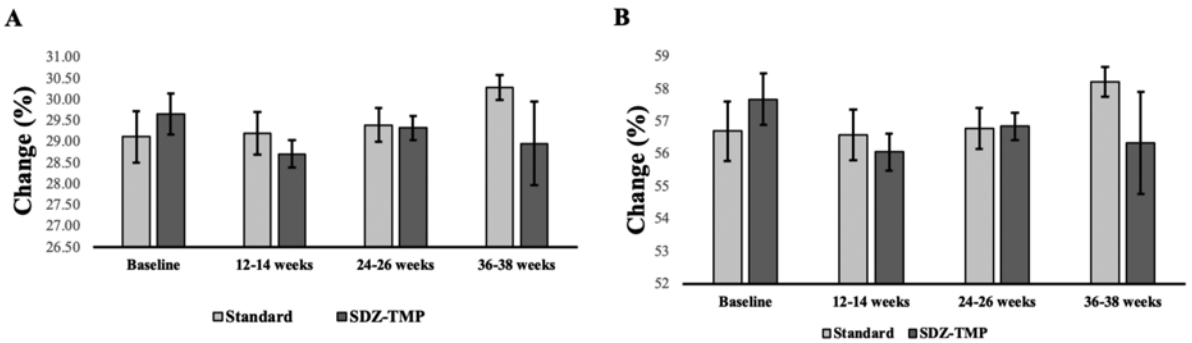

Figure 1. Mean (± SE) values for (A) fractional shortening and (B) ejection fraction over time. No significant difference in LV function was observed between diet groups.

whereas mice fed SDZ-TMP diet had gained approximately 6.9 grams. This difference in weight gain between the 2 diet groups was significant ($P < 0.01$). Moreover, males gained more weight over time than did females (Table 8; $P < 0.01$).

Gross necropsy and histopathology. Hearts from the 5 animals that died or were euthanized prior to the end of the study were subjectively normal and showed no histologic evidence of cardiomyopathy. At the end of the study, hearts from the remaining 38 mice appeared subjectively normal on gross necropsy. In addition to decreased cardiac function, SW-20 had a subjectively enlarged heart. Ventricular wall thickness was not measured histologically, although when examined subjectively, the ventricles of 4 SW mice (3 females, 1 male) on SDZ-TMP diet, including SW-20, appeared thin. On histologic evaluation, large round vacuoles, suggestive of lipid droplets were seen in the cardiomyocytes of many animals. Table 1 describes the numerical scale used to grade the severity of microscopic lesions. SW-20 had marked degeneration, moderate fibrosis, and inflammation in all areas of the heart evaluated, suggestive of cardiomyopathy. All other mice had mild to moderate changes in the areas described, but none were suggestive of cardiomyopathy histologically. As stated previously, the sum of the heart scores for Hsd:ICR (CD1) and Tac:SW were not combined as the other parameters due to the large variability seen within the SW groups. Statistical analysis was not performed to compare the 2 stocks; however, numerically, SW mice had a higher score for cardiomyocyte degeneration and fibrosis then ICR mice (data not shown). For both outbred stocks, heart scores did not differ significantly between the diet groups (Table 9 and Figure 3).

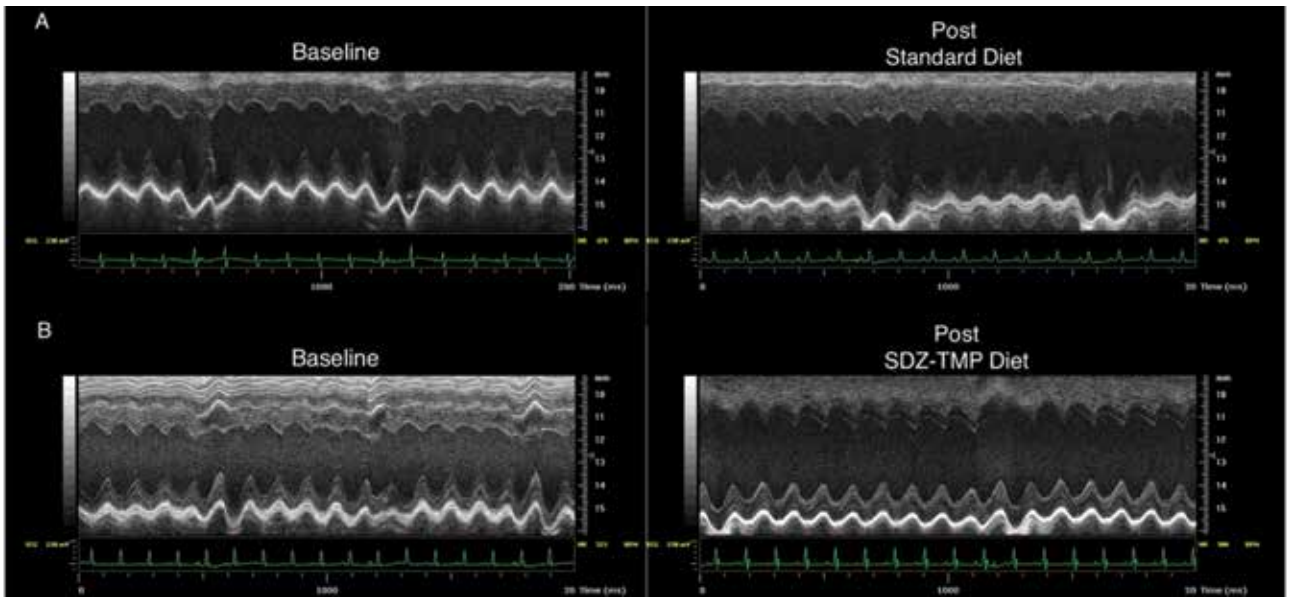

Figure 2. Representative M-mode images from mice in both diet groups. No functional difference between (A) standard diet or (B) SDZ-TMP diet was detected after 36 to 38 wk on the diet

Table 4. Differences in cardiac function were not significant between the standard and SDZ-TMP diet groups.

	Delta 1 (2 sample *t* test)	Delta 2 (2 sample *t* test)	Mixed-effect regression model (effect of diet)
Fractional shortening	t(40) = 1.2930, $P = 0.2034$	t(37) = 1.3064, $P = 0.1995$	$P = 0.164$
Ejection fraction	t(40) = 1.3074, $P = 0.1986$	t(37) = 1.3589, $P = 0.1824$	$P = 0.185$
LVIDd	t(40) = 1.4300, $P = 0.1605$	t(37) = 0.4941, $P = 0.6242$	$P = 0.109$

Table 5. CBC parameters associated with RBCs as well as values for platelets (mean ± 1 SD).

	Baseline	14–16 wk	40–42 wk
Standard diet RBC, $10^3/\mu L$	9.99 ± 0.58	10.58 ± 0.83	9.77 ± 0.90
SDZ-TMP diet RBC, $10^3/\mu L$	10.09 ± 0.67	9.99 ± 1.32	9.74 ± 1.14
Standard diet hemoglobin, (g/dL)	16.65 ± 0.72	16.13 ± 0.97	14.95 ± 1.66
SDZ-TMP diet hemoglobin, (g/dL)	16.56 ± 0.92	15.1 ± 2.09	14.77 ± 1.88
Standard diet hematocrit, (%)	56.18 ± 2.91	56.55 ± 3.37	50.98 ± 5.12
SDZ-TMP diet hematocrit, (%)	55.96 ± 3.36	52.60 ± 7.88*	49.70 ± 5.91
Standard diet MCV, fL	56.25 ± 1.86	53.61 ± 2.78	52.22 ± 3.46
SDZ-TMP diet MCV, fL	55.55 ± 2.51	52.51 ± 2.69	51.08 ± 2.63
Standard diet MCH, g/dL	16.69 ± 0.63	15.31 ± 1.01	15.31 ± 1.17
SDZ-TMP diet MCH, g/dL	16.44 ± 0.74	15.13 ± 0.85	15.17 ± 0.94
Standard diet MCHC, g/dL	29.67 ± 0.99	28.55 ± 1.21	29.31 ± 0.81
SDZ-TMP diet MCHC, g/dL	29.63 ± 1.45	28.84 ± 1.66	29.71 ± 1.29
Standard diet platelets, $10^3/\mu L$	1126.86 ± 220.09	1108.40 ± 374.99	1288.61 ± 373.53[a]
SDZ-TMP diet platelets, $10^3/\mu L$	1124.09 ± 258.86	1052.68 ± 312.19	1051.14 ± 369.52[a]
Standard diet reticulocytes, $10^3/\mu L$	351.64 ± 76.74	407.94 ± 101.09	504.37 ± 147.02[b]
SDZ-TMP diet reticulocytes, $10^3/\mu L$	336.39 ± 97.47	398.04 ± 135.35	398.05 ± 121.68[b]

Many parameters showed significant differences over time, but most means fell within their reference range.[18] Only values that differed between the 2 diet groups are marked with the same superscript ($P < 0.05$). Platelets decreased significantly in the SDZ-TMP diet group,[a] and reticulocytes were significantly higher in the standard diet group.[b] SW mouse fed SDZ-TMP medicated diet was anemic (23.9%) at 14-16 wk, therefore the higher standard deviation.* Anemia resolved by 36-38 wk (48.5%).

All lungs were examined and appeared grossly normal, except for 2 mice with visible, white, circular lesions present within their lungs. Histologic evaluation revealed a multifocal granulomatous interstitial pneumonia for one mouse (SW male, standard diet) and a pulmonary adenoma for the other mouse (ICR female, standard diet). No foreign material or infectious agent were identified in the lungs of the mouse with the granulomatous pneumonia, even after an acid-fast stain. Two additional male mice (standard diet), including one ICR and one SW, had pulmonary adenomas on histologic analysis. A focal pulmonary carcinoma was found in one female SW mouse in the standard diet group. This mouse also had a sizeable retroperitoneal mass on gross necropsy, which was consistent with a cystic lymph node histologically.

Table 6. CBC parameters for WBC (mean ± 1 SD)

	Baseline	14–16 wk	40–42 wk
Standard diet WBC, $10^3/\mu L$	5.16 ± 1.15	7.75 ± 1.97	5.27 ± 2.71[a]
SDZ-TMP diet WBC, $10^3/\mu L$	4.74 ± 0.96	5.54 ± 1.91	3.93 ± 1.28[a]
Standard diet neutrophils, $10^3/\mu L$	0.87 ± 0.40	1.55 ± 1.25	1.20 ± 0.60
SDZ-TMP diet neutrophils, $10^3/\mu L$	0.77 ± 0.22	1.29 ± 1.05	1.04 ± 0.54
Standard diet lymphocytes, $10^3/\mu L$	4.11 ± 1.00	5.94 ± 1.62	3.70 ± 2.16[b]
SDZ-TMP diet lymphocytes, $10^3/\mu L$	3.80 ± 0.88	4.03 ± 1.45	2.59 ± 1.07[b]
Standard diet monocytes, $10^3/\mu L$	0.03 ± 0.01	0.10 ± 0.06	0.22 ± 0.18[c]
SDZ-TMP diet monocytes, $10^3/\mu L$	0.03 ± 0.02	0.07 ± 0.06	0.15 ± 0.15[c]
Standard diet eosinophils, $10^3/\mu L$	0.14 ± 0.06	0.15 ± 0.03	0.14 ± 0.09
SDZ-TMP diet eosinophils, $10^3/\mu L$	0.13 ± 0.07	0.13 ± 0.07	0.14 ± 0.09
Standard diet basophils, $10^3/\mu L$	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.02
SDZ-TMP diet basophils, $10^3/\mu L$	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01

Values with the same superscript letter differ significantly between the 2 diet groups ($P < 0.05$). A significant decline in WBCs and lymphocytes was seen in the SDZ-TMP diet group,[a,b] whereas monocytes increased in both diet groups, but the increase was greater in mice fed standard diet.[c]

Table 7. Serum biochemistry values (mean ± 1 SD)

	Baseline	14–16 wk	40–42 wk
Standard diet ALT, U/L	41.60 ± 11.70	54.85 ± 30.53	44.35 ± 16.80
SDZ-TMP diet ALT, U/L	48.83 ± 17.94	44.43 ± 23.88	49.80 ± 27.26
Standard diet AST, U/L	148.20 ± 125.29	159.60 ± 169.17	90.29 ± 29.35
SDZ-TMP diet AST, U/L	134.91 ± 79.66	100.86 ± 82.90	77.80 ± 39.58
Standard diet BUN, mg/dL	21.50 ± 3.49	24.90 ± 4.29	22.41 ± 6.49[a]
SDZ-TMP diet BUN, mg/dL	20.74 ± 2.85	19.27 ± 3.82	21.45 ± 6.03[a]
Standard diet creatinine, mg/dL	0.30 ± 0.07	0.20 ± 0.02	0.20 ± 5.71E-17
SDZ-TMP diet creatinine, mg/dL	0.30 ± 0.09	0.22 ± 0.04	0.21 ± 0.05
Standard diet calcium, mg/dL	9.21 ± 0.24	9.13 ± 0.40	9.36 ± 0.48
SDZ-TMP diet calcium, mg/dL	9.45 ± 0.22	9.51 ± 0.39	9.44 ± 0.55
Standard diet magnesium, mg/dL	2.33 ± 0.21	2.79 ± 0.48	2.76 ± 0.33
SDZ-TMP diet magnesium, mg/dL	2.46 ± 0.22	2.81 ± 0.61	2.64 ± 0.30
Standard diet creatine kinase, U/L	759.95 ± 705.36	657.58 ± 585.54	392.18 ± 309.68
SDZ-TMP diet creatine kinase, U/L	934.30 ± 811.74	777.73 ± 1078.86	439.30 ± 658.66

Creatinine, magnesium and creatine kinase showed significant differences over time, but the means fell within their reference range. BUN was the only parameter that differed between the 2 diet groups ($P < 0.05$).[a]

Table 8. Mean (± 1 SD) body weight (grams) for male and female mice fed either standard diet or SDZ-TMP diet.

	Baseline	12 wk	24 wk	36 wk
Standard diet males	35.61 ± 5.35	46.12 ± 9.00	50.53 ± 10.06	52.06 ± 7.71
SDZ-TMP diet males	35.36 ± 4.13	39.73 ± 8.64	42.81 ± 8.72	43.18 ± 9.26
Standard diet females	28.18 ± 4.30	36.56 ± 9.40	36.59 ± 10.63	36.62 ± 9.91
SDZ-TMP diet females	27.16 ± 2.45	30.53 ± 2.69	32.84 ± 3.04	33.31 ± 5.19

Males gained significantly more weight than females over time ($P < 0.01$).

Table 9. Mean (± 1 SD) sum of heart scores for both outbred stocks either fed standard or SDZ-TMP diet.

Standard diet ICR	SDZ-TMP diet ICR	Standard diet SW	SDZ-TMP diet SW
5.00 ± 2.4	4.44 ± 1.67	10.25 ± 7.96	11.72 ± 8.40

No significant difference was seen between mice fed standard or SDZ-TMP diet for either stock.

Femurs were submitted for histologic assessment of bone marrow. The marrow cavity of all mice was well populated with cells from all lineages, except for one SW male on SDZ-TMP diet. The marrow cavity of this mouse was populated predominately with adipose tissue and sparsely by hematopoietic cells, although all cell lineages were represented.

Other gross findings included a colon mass ($n = 1$; SW female, SDZ-TMP diet), thickened uterine horns ($n = 1$; ICR female, standard diet), and irregular kidneys ($n = 2$; SW male, SDZ-TMP diet; SW female, SDZ-TMP diet). On histology, the colon mass was consistent with a histiocytic sarcoma, the thickened uterine horns were due to bilateral endometrial hyperplasia,

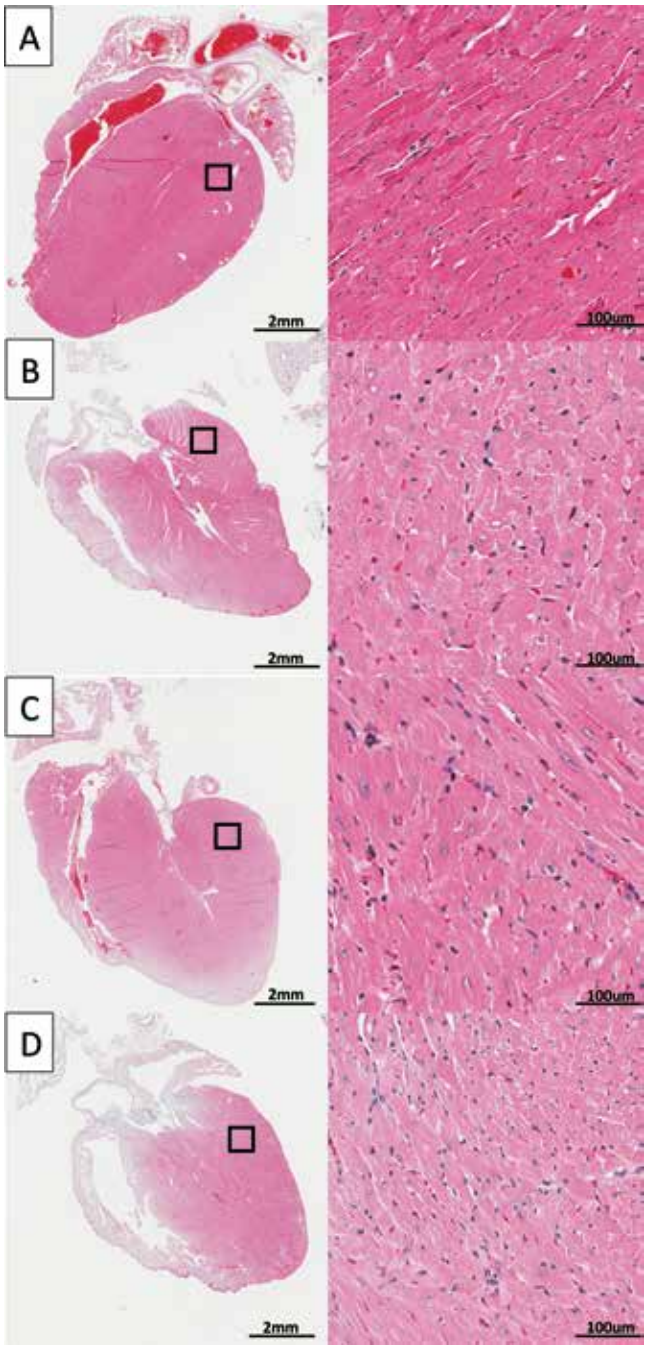

Figure 3. Representative pictomicrographs of hearts from ICR mouse fed standard diet (A), ICR mouse fed SDZ-TMP diet (B), SW mouse fed standard diet (C), and SW mouse fed SDZ-TMP diet (D). Regions indicated by square are shown in higher magnification next to each heart. Amount of cardiomyocyte degeneration, and fibrosis was not significant between the 2 diet groups in either outbred stock. Hematoxylin and eosin stain; magnification 2× and 5×.

and the kidney irregularities were associated with moderate multifocal perivascular mononuclear cell infiltration, with mild mineralization.

Discussion

Rodents, particularly mice, are frequently used as a model for heart disease due to their size, short gestation, and lower maintenance costs as compared with larger animals.[44] The goal of our study was to develop a low-cost model of cardiomyopathy without the need for surgery by using a commonly administered rodent antibiotic diet made with SDZ-TMP. Previous serendipitous findings at our institution showed that IcrTac:ICR mice, when fed SDZ-TMP medicated diet for 3 to 6 mo, developed cardiomyopathy and cardiac decompensation.[48] Therefore, we hypothesized that mice fed SDZ-TMP medicated diet continuously for 3 to 6 mo would develop cardiomyocyte degeneration and fibrosis, eventually leading to dilated cardiomyopathy. However, we did not reproduce these findings in either of the outbred stocks (ICR and SW) used for this study. The findings showed no significant differences in FS, EF, or LVIDd between the 2 diet groups, thus negating our hypothesis. LVIDd increased over time in all mice, and females had a shorter LVIDd than males. This sex difference is consistent with published reports in humans[36,49] and is likely due to the smaller size of females. Left ventricular internal diameter is used to assess left ventricular size and to determine if the ventricle is dilated. The severity of ventricular dilation can be used as a risk marker for sudden cardiovascular death in people with decreased cardiac function.[46] The significance of the increase observed in these mice over time is likely nominal, as cardiac function in both diet groups remained constant throughout the study. Furthermore, the increase in LVIDd was evident at the 12 to 14-wk time point, suggesting an age-related finding as these animals were approximately 5 wks of age at baseline.

SDZ-TMP is a potentiated sulfonamide with a broad spectrum of activity and is commonly used in both veterinary and human medicine. It has been associated with adverse side effects such as keratitis sicca, hypothyroidism, as well as bone marrow suppression and hypersensitivity reactions leading to anemia and leukopenia.[21,50] Due to these adverse effects, we wanted to investigate the effects of its long-term use in a medicated diet in mice and document any changes in hematology, select chemistry parameters, and weight gain. Moreover, we wanted to explore any potential correlation with changes in these parameters with cardiac dysfunction.

SDZ-TMP medicated diet did not have a significant effect on RBCs, hemoglobin, hematocrit, neutrophils, eosinophils, or basophils. A decrease in hemoglobin and hematocrit occurred over time in all mice, consistent with other published reports.[6,13,18,22] Previous studies have shown that this decrease is due to plasma volume expansion and not an actual decrease in RBCs.[6,18,40] Despite the decrease in hemoglobin and hematocrit, the majority of the study mice in both diet groups did not have values consistent with anemia, except for 2 SW females in the SDZ-TMP diet group. The first mouse had a 23% HCT at the 14 to 16-wk time point; however, when CBC parameters were rechecked 2 wk later, the anemia had resolved, as the mouse had a 52% HCT. In addition, the HCT was within normal limits at the final time point of the study. The second mouse with anemia was SW-20; the HCT of this mouse decreased from 45% (14 to 16 wk) to 33% (36 to 38 wk).

Platelets decreased significantly ($P < 0.02$) in SDZ-TMP diet group (1124 ± 259 (baseline); 1051 ± 370 (36 to 38 wk)); however, values for all animals remained within published reference ranges for mice.[18] Low platelet counts can be due to decreased production or shortened half-life caused by immune mediated injury to megakaryocytes or peripheral platelets.[18] Prolonged administration of sulfonamides, including SDZ-TMP, has been associated with immune mediated thrombocytopenia.[21] On histology, the bone marrow cavity of all animals was well populated with cells from all lineages, including megakaryocytes, suggesting that a SDZ-TMP mediated immune effect was unlikely; however, cytology may have provided more insight on cellular details and precursors.

Over time, reticulocyte numbers increased in both diet groups compared with baseline, but the increase was greater in the standard diet group. Erythropoietin, a hormone primarily produced by the kidneys to stimulate red blood cell production, was not measured in this study; therefore, we do not know if SDZ-TMP had a negative effect on this hormone. However, kidney values, including BUN and creatinine, did not show evidence of kidney dysfunction, suggesting that erythropoietin was unlikely to be affected by the SDZ-TMP medicated diet. Furthermore, blood samples for CBC were taken approximately 1 wk after retroorbital blood collection for chemistry analysis. Although the sample did not exceed 10% of the total circulating blood volume, the prior week's blood collection may have stimulated the production of reticulocytes.

A significant decline occurred over time in the WBC of mice fed SDZ-TMP medicated diet, as compared with the standard diet, but no indication of leukopenia or bone marrow toxicity was detected at the end of the study. Studies have shown that broad-spectrum antibiotics can decrease bacterial load in 2 wk or less in mice.[2,8,26] Potentially, the mice fed SDZ-TMP medicated diet had a lower microbial burden due to the constant oral administration of antibiotics, which may have led to a lower WBC in this diet group. Future studies are needed to quantify the effects of chronic antibiotic administration on the gastrointestinal bacterial load. In addition, mice in the SDZ-TMP group did not have any gross or histologic lung lesions as compared with mice in the standard group. The lack of these findings in mice fed SDZ-TMP medicated diet may have also contributed to the lower leukocyte count, as these lesions were likely associated with an inflammatory response, therefore increasing the WBC of mice in the standard diet group. In contrast to our study hypothesis, one study found that mice with induced heart failure and treated with a cocktail of broad-spectrum antibiotics had improved cardiac function and decreased cardiac fibrosis, likely due to a reduction in T cells and inflammatory cytokines.[11]

Long-term use of SDZ-TMP medicated diet did not reveal signs of liver damage, as no increases occurred in the enzymes ALT or AST. It also did not affect serum calcium, magnesium, or creatine kinase. Both calcium and magnesium are needed for muscle contraction, such as cardiomyocyte function, and low levels of these minerals have been associated with arrhythmias, impaired cardiac contractility, and heart failure.[1,5,12,17,19,29,34,42,51,60,62,65] On the other hand, creatine kinase is found in numerous tissues, including the heart, and is released in response to muscle damage. Therefore, we monitored these parameters in case of a potential mechanism or link with SDZ-TMP medicated diet.

Predictably, mice in both diet groups gained weight over time, but the increase was higher in mice fed the standard diet. Why mice in the standard diet group gained more weight than those fed SDZ-TMP medicated diet is uncertain, although the palatability of the medicated diet has not been studied. However, the antibiotics may have altered the gut microbiota, in turn influencing nutrient metabolism and absorption. In addition, despite a lower weight gain, mice in the SDZ-TMP diet group that achieved the final endpoint of the study had normal body condition scores (data not shown) and appeared to be in good health.

Cardiomyopathy, defined by decreased cardiac function and histologic changes associated with severe cardiomyocyte degeneration and fibrosis, was seen in one SW female mouse in the SDZ-TMP diet group (SW-20). In all other mice, the number and degree of histologic lesions observed in cardiac tissue did not differ between the 2 diet groups. For SW-20, cardiac dysfunction was first detected by echocardiography at approximately 9 mo after the start of SDZ-TMP medicated diet. In addition, SW-20's final CBC revealed a normocytic normochromic anemia with a total RBC of $5.98 \times 10^3/\mu L$ and HCT of 33%. SDZ-TMP has been associated with anemia when administered long term, and anemia can potentially lead to cardiomyopathy. How anemia leads to cardiomyopathy and heart failure is not completely understood, but a possibility is that heart enlargement occurs due to overcompensation from a lack of oxygen in the blood. Alternatively, congestive heart failure can lead to anemia through various and complex pathways including a decrease in erythropoietin due to renal dysfunction, or from chronic inflammation leading to iron deficiency or erythropoietin resistance.[10,28,35,43,45,61,63,64,67] Histologically, the bone marrow cavity of this animal was well populated by cells from all lineages. Therefore, SDZ-TMP did not likely contribute to bone marrow toxicity and it is possible that the anemia was a consequence of heart failure.

Limitations of this study include the lack of inclusion of ICRs from Taconic, not evaluating bone marrow via cytology or flow cytometry, and the number of animals lost to lymphoma prior to the end of the study. As stated previously, live production of IcrTac:ICR ceased but communication with the vendor indicated no reports of unexpected cardiomyopathy in their ICR colony prior to this decision, and therefore they recommended replacement with Tac:SW. The outbred stock, ICR, was initially created at the Institute of Cancer Research (Philadelphia, PA) from a colony of Swiss mice at the Rockefeller Institute (New York, NY).[32,41,56] From there, ICRs were distributed to multiple vendors including Taconic and Harlan, who maintain or have maintained their own breeding colony for decades.[56] Even though IcrTac:ICR and Hsd:ICR (CD1) mice share a similar genetic background, differences from the 2 vendors are presumed, which may have led to the irreproducibility of this model. Second, cytology provides better cellular details and differentiation of cell types than histology. The use of flow cytometry would have provided more information on the cell populations and any characteristic changes. Finally, 44 animals were initially enrolled in the study, with the expectation that only 1 or 2 would die, or require euthanasia prior to study endpoint. Unfortunately, 5 mice were lost prior to the end of the study, including 3 that died from lymphoma. Two of these mice were on SDZ-TMP diet for approximately 4 mo before their death; however, no correlation can be made based on such a small number. Lymphoma is the most common neoplasm of the hematopoietic system, but it is more commonly seen in mice over one year of age.[7,59]

In summary, we were not able to reproduce our previous findings [48] and the current results do not support our hypothesis because mice fed SDZ-TMP medicated diet did not develop cardiomyopathy. We did observe the development of cardiomyopathy in one mouse fed SDZ-TMP, but a correlation cannot be made based on only one mouse. Furthermore, SDZ-TMP medicated diet did not significantly change RBC parameters, hematopoiesis or the selected biochemistry parameters such as AST, ALT, and creatinine. Even though platelets and WBCs decreased significantly over time in mice fed SDZ-TMP medicated diet, values were clinically insignificant because they remained within published reference ranges.[18] Mice fed SDZ-TMP medicated diet gained less weight by the end of the study, but they remained in good body condition. Overall, the findings presented here reveal that SDZ-TMP medicated diet, containing 1,365 ppm of SDZ and 275 ppm of TMP appears to be safe for mice, even long term. This information is useful for lab animal veterinarians, technicians and investigative staff who need to maintain animals on long-term antibiotics for various reasons including immune compromise.

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