MEDICAL COUNTERMEASURES FOR THE TREATMENT OF BRODIFACOUM (BDF) POISONING

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ABSTRACT

Superwarfarins (SWs) are modified forms of the commonly used anti coagulant Warfarin, and cause bleeding by reducing vitamin K levels. SWs are used throughout the world as rodenticides. The SW most commonly currently used is brodifacoum (BDF). BDF is about 100 times more potent than warfarin, because it is more fatty and stays in the body longer. In addition, it appears that the biological half life of BDF is increased due to enterohepatic recirculation (EH). SWs like BDF not only block clotting but also accumulate in the CNS where they can induce long lasting neuropathology. We are therefore developing a model of BDF poisoning in rabbits in order to identify novel treatments to reduce acute toxicity and long term consequences.

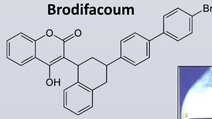
To determine the LD_{50} of BDF in adult male NZW rabbits, animals were administered a single oral dose of BDF ranging from 0.050 to 0.300 mg /kg, after which survival was followed out to 2 weeks. Calculation of the LD_{50} was done using the Dixon up-down method and resulted in an LD_{50} dose of 0.192 ± 29 mg/kg. Mean body temperature decreased following BDF, and the body temperature of each animal decreased to below 98ºC one day prior to the animal being classified as moribund. BDF reduced red blood cell count and total hematocrit, consistent with induction of hemorrhage. BDF administration at 0.2 mg/kg resulted in 70% mortality and a time-dependent increase in Prothrombin Time (PT). HPLC/MS-MS analysis showed time-dependent accumulation of BDF in all tissues including brain and spinal cord, with highest levels in liver. Serum BDF levels peaked at 24 hr after administration.

Two medical countermeasures to BDF poisoning were evaluated, the lipid emulsion Intralipid (IL) and the bile sequestrant cholestyramine (CSA). IL is thought to act by scavenging toxins and moving them to the liver where they are metabolized. CSA is known to block EH recirculation by binding to the bile in the intestines, therefore increasing elimination from the intestines. IL (30%) was administered at 2 hr, Day 3 and Day 5 after BDF by intravenous infusion at 0.2 mg/kg; CSA was administered once daily for 14 days after BDF administration. Administration of IL or CSA resulted in a decrease in mortality to 33% and 11%, respectively. In addition, IL and CSA administration both resulted in a decrease in PT at Day 10 by 42% and 70%, respectively.

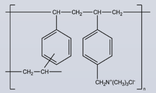
Immunostaining of brain cortical sections from vehicle and BDF treated rabbits revealed an overall decrease in the number of ramified microglia cells, indicative of conversion to an activated morphology. Brain lipid content was measured as an index of oxidative stress. After 15 days, treatment with BDF caused a small, but statistically significant increase in cortical and cerebellar cholesterol content as compared to the vehicle treated group. Effects of IL and CSA on brain pathology is in progress.

In conclusion, both IL and CSA are potential medical countermeasures against BDF poisoning in humans, justifying more extensive evaluations.

Brodifacoum



Cholestyramine



Intralipid

OBJECTIVES

Poisoning by a SW requires daily Vitamin K for up to a year, and if interrupted can cause a recurrence of symptoms. Also, while Vitamin K treats the problem, it does not remove the toxin from the body. The goal of our research is to develop countermeasures to prevent or reduce BDF toxicity. For this we are testing the ability of the FDA approved drugs, Intralipid and cholestyramine to counteract the effects of BDF poisoning.

The current research is to optimize treatment with Intralipid to achieve maximal survival, and also to confirm that the FDA approved 20% intralipid is as effective as the 30% intralipid that we have been using (which is not FDA approved). In addition, we have determined that the biological half life of BDF is increased due to enterohepatic recirculation (EH). We will therefore test if another FDA approved drug, cholestyramine (CSA) which is known to block EH recirculation, further increases survival.

MATERIALS AND METHODS

Animals received a single gavage dose of BDF (0.1 mg/mL) at a dose volume of 2 mL/kg to provide a final total dose of 0.20 mg/kg.

Intralipid was infused over a 2 hour period into the marginal ear vein of each rabbit at 2 hr, Day 3, and Day 5 after BDF administration

For treatment with Intralipid, the animals were placed into a custom restraint jacket (Lomir Biomedical) to house the ambulatory pump during the infusion period. An intravascular catheter placed in the lateral ear vein and extension tubing was connected between the pump and the catheter to deliver the intralipid. The animals were also fitted with an Elizabethan collar for the 2-hour duration of the infusion.

Cholestyramine was suspended in 20 mL of water. Animals in Groups 3 and 5 received cholestyramine by oral gavage (2 gm in 20 mL water on Day 1 at 2 hours after BDF administration and then once daily thereafter until Day 15.

A blood sample (~1.8 mL) was collected from the medial ear artery of each rabbit in pretest, and on Days 2, 8, 10, 15 in surviving rabbits to monitor coagulation changes (APTT, PT, and fibrinogen) following BDF administration.

Cholesterol levels in brain regions was measured using a fluorescent assay kit (Sigma) in which cholesterol is esterified to a fluorescent product. Tissue samples were prepared from a portion of the frontal cortex and from the cerebellum.

Immunostaining for microglial activation was carried in sagittal sections prepared from paraffin embedded, 4% PFA post fixed brains. The rabbit anti-Iba1 (Sigma Chemical Company) antibody detects a calcium binding protein expressed in macrophages and microglia cells.

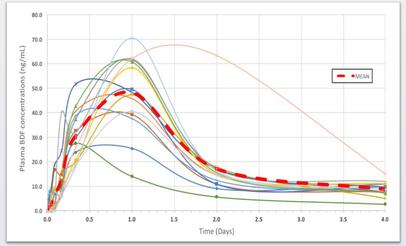
EXPERIMENTAL DESIGN

Group	Number of Animals	Cholestyramine Dose (g)	Lipid Dose Volume (mL/kg)	BDF Dose (mg/kg)
1 @	3	N/A	N/A	N/A
2	6	0 #	0	0.2
3	10	2	0	0.2
4	8	0 #	12	0.2
5	3	2	12	0.2

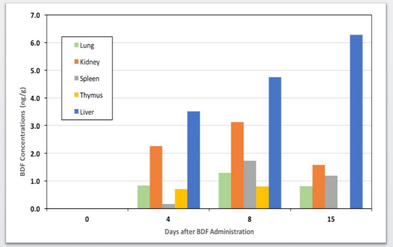
@ Group 1 was a naïve sham control group that did not receive BDF or any treatment.
# Water was administered at the same dose volume (20 mL) as cholestyramine.

RESULTS

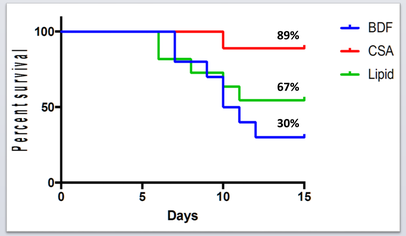
Individual and Mean Plasma Concentrations Following Oral Administration of BDF



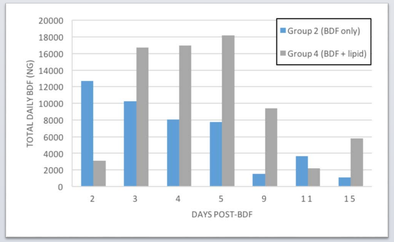
Mean Concentrations of BDF in Selected Tissues Following BDF Administration



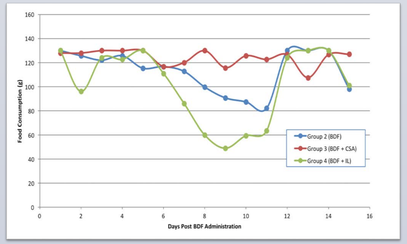
Kaplan Meier Plot of Survival for 15 Days Following BDF Administration after Treatment with intralipid or cholestyramine



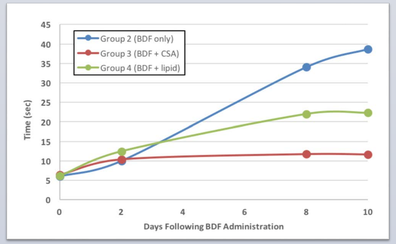
Mean Fecal BDF Levels Following BDF Administration and Treatment with Intralipid



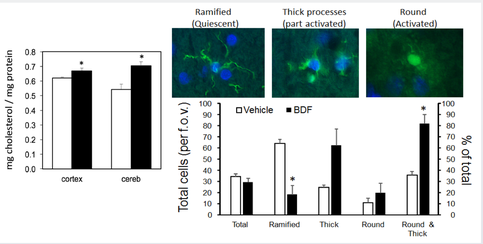
Mean Food Consumption Following BDF Administration and Treatment with either Intralipid or cholestyramine



Mean Prothrombin Time (PT) Following BDF Administration and Treatment with Intralipid or cholestyramine



Brain Lipid Content was Measured as an Index of Oxidative Stress and Immunostaining of Cortical sections of Vehicle and BDF treated NZW Rabbits Following BDF Administration Showing Ramified Microglial Cells. Data is presented in Mean ± SE of samples, *, P < 0.05 versus treated rabbits (t-test)



CONCLUSIONS

Adult male NZW rabbits were administered a single oral dose of BDF ranging from 0.050 to 0.300 mg/kg, after which survival was followed out to 2 weeks. Calculation of LD50 was done using the Dixon up-down method and gave 192 ± 29 ug/kg. Necropsy performed on moribund sacrificed rabbits revealed massive internal hemorrhages in the mediastinum, as well as some minor hemorrhage in the lungs. Measurement of PT (Prothrombin time) showed a time dependent increase due to BDF. Mean body temperature decrease following BDF, and a temperature below 100°F occurred 1 day before death. BDF reduced red blood cell count and total hematocrit, consistent with induction of hemorrhage.

Intralipid administered as a slow IV infusion (12 mL/kg of 30% IL, @ 0.10 mL/min) at 2 hr, 3 days, and 5 days after BDF (given at 0.20 mg/kg, slightly higher than LD50) reduced mortality. In the control group, 7 of 10 (70%) rabbits died within 2 weeks. In the Lipid group, only 3 of 9 (33%) rabbits died. Intralipid reduced the increase in PT time, reduced body temperature decrease due to BDF, and reduced loss of RBCs and hematocrit. The mechanism of action of Intralipid is not clear, however measurements of fecal excretion show an increased elimination of BDF due to Intralipid. When CSA was administered to rabbits once daily beginning 2 hr after BDF administration mortality was reduced to 1 of 9 (11%). Administration of CSA resulted in significant reduction in the anticoagulation effects of BDF (only mild increases in PT time were seen following BDF administration). Fecal excretion could not be measured in the CSA group due to an inability to separate the BDF bound to the CSA with the current analytical method.

Immunostaining of cortical sections of vehicle and BDF treated NZW rabbits revealed an overall decrease in the number of ramified microglia cells, and conversion to an activated morphology (fewer and shorter cell processes). Brain lipid content was measured as an index of oxidative stress. After 15 days, treatment with BDF caused a small, but statistically significant increase in cortical and cerebellar cholesterol content as compared to the vehicle treated group. Our previous findings of reduced cholesterol (rats and rabbits) were performed at earlier time points (4-7 days) suggesting a delayed and compensatory increase following cholesterol depletion.

In conclusion, CSA and Intralipid reduced mortality in rabbits following administration of an LD70 dose of BDF. Further testing to compare these treatments along with the current standard of care treatment regimen of oral Vitamin K is planned for future experiments.

ACKNOWLEDGMENT

This project is funded by the NIH Counter Act Program (U01 NS083457-01)