

# Formoterol fumarate and roxithromycin effects on muscle mass in an animal model of cancer cachexia

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Received December 20, 2007; Accepted January 24, 2008

**Abstract.** Our study probed the effects of the  $\beta$ -2 adrenergic agonist, formoterol and the macrolide antibiotic, roxithromycin, on muscle wasting in a well-characterized animal model of cancer cachexia. Female Wistar rats were inoculated with Yoshida AH130 ascites hepatoma (AH) cells to induce rapid and severe cachexia as demonstrated by wet weight determinations of the hearts, gastrocnemius muscles and carcasses. The control animals received saline (vehicle) inoculations. The AH-inoculated rats were treated once daily for four days by i.p. injection with a vehicle control, 1 mg/kg formoterol, 5 and 50 mg/kg roxithromycin or 1 mg/kg formoterol plus 5, 25, 40 and 50 mg/kg roxithromycin. The saline-inoculated animals were treated by i.p. injection with vehicle control, 1 mg/kg formoterol, 5 and 40 mg/kg roxithromycin. As a result, formoterol alone reduced the loss of muscle mass in the AH-inoculated rats by approximately one-half, consistent with literature reports. Roxithromycin alone at 5 mg/kg did not affect muscle mass in the AH-inoculated rats. Roxithromycin given alone at 50 mg/kg reduced the loss of muscle mass in AH-inoculated animals by approximately one-half. With respect to the antagonizing muscle loss, formoterol combined with either 5 or 25 mg/kg roxithromycin did not reach statistical significance versus formoterol alone, while formoterol plus either 40 or 50 mg/kg roxithromycin enhanced protection against muscle loss versus formoterol alone. The gastrocnemius weights in the AH-inoculated rats treated with formoterol combined with 40 mg/kg roxithromycin were not significantly different from the muscle weights in the saline-inoculated controls. To sum up, formoterol and roxithromycin apparently exert anti-cachectic effects in an additive fashion and may offer the potential for combination therapy in cachexia.

## Introduction

Patients with advanced malignancies frequently develop anorexia and cachexia (1-14). Anorexia, a medical term for appetite loss, includes a decreased sense of taste and smell for food, early satiety, a decreased sense of hunger and outright aversion to food. Catabolic wasting, or cachexia, is a syndrome characterized by the involuntary and progressive loss of fat and skeletal muscle, refractoriness of weight loss to an increased nutritional input, an elevated resting energy expenditure (REE), a decreased protein synthesis, an altered carbohydrate metabolism (increased *Cori* cycle activity), hypercatabolism of the muscle via elevated proteolysis and of the adipose tissue via lipolysis (13,15). At least 5% of pre-illness body weight must have been lost before a patient is diagnosed with cachexia. Roughly half of all cancer patients experience some degree of catabolic wasting, with a higher occurrence seen in cases of malignancies of the lung, pancreas and gastrointestinal tract (16). The syndrome is found in patients with immunodeficiency disorders, such as AIDS, as well patients suffering from bacterial and parasitic diseases, rheumatoid arthritis and chronic diseases of the bowel, liver, kidneys, lungs and heart. Cachexia is often associated with anorexia and can manifest as a condition in aging or as a result of physical trauma and burn injuries. The cachexia syndrome diminishes the functional ability and quality of life of the patient, worsens the underlying condition, reduces tolerance to medications and is a major cause of death.

Despite the fact that cachexia is often associated with cancer, no consistent relationship has been demonstrated between the development of cachexia and tumor size, disease stage and the type or duration of the malignancy. Molecular mechanisms underlying the cancer anorexia and cachexia syndrome (CACS) are complex and incompletely understood. Elevated levels of pro-inflammatory cytokines (such as interleukin-6 and tumor necrosis factor alpha) and acute phase proteins (such as the C-reactive protein) are a host response to the malignancy and a trigger mechanism for metabolic abnormalities such as muscle hypercatabolism (17-21). The ATP-ubiquitin-dependent proteasome pathway of proteolysis is a well-accepted (12,22-26) mechanism in cachexia. Numerous studies have demonstrated that ubiquitin and proteasomal

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**Key words:** formoterol, roxithromycin, AH130, rat, cachexia

Table I. Experimental design for rat ascites hepatoma (AH) studies with roxithromycin and formoterol treatment groups.<sup>a</sup>

Experiment no.	Group no.	Inoculum	Intraperitoneal dose (mg/kg)	
			Formoterol fumarate	Roxithromycin
1	1	AH <sup>b</sup>	0	0
1	2	Saline	0	0
1	3	AH <sup>b</sup>	1	0
1	4	AH <sup>b</sup>	0	5
2	5	AH <sup>b</sup>	0	0
2	6	Saline	0	0
2	7	AH <sup>b</sup>	1	0
2	8	AH <sup>b</sup>	1	5
2	9	Saline	0	5
2	10	Saline	1	0
3	11	AH <sup>b</sup>	0	0
3	12	Saline	0	0
3	13	AH <sup>b</sup>	1	0
3	14	AH <sup>b</sup>	1	40
3	15	Saline	0	40
4	16	AH <sup>b</sup>	0	0
4	17	Saline	0	0
4	18	AH <sup>b</sup>	1	0
4	19	AH <sup>b</sup>	1	25
4	20	AH <sup>b</sup>	1	50
4	21	AH <sup>b</sup>	0	50

<sup>a</sup>Treatment Group = Inoculation type/vehicle, or treatment group = Inoculation type/F(0)R(0), where, inoculation type is either AH or saline; F, formoterol fumarate; R, roxithromycin and the parenthetical values are formoterol fumarate and roxithromycin doses (i.p. in mg/kg).

<sup>b</sup>2.0x10<sup>7</sup> cells/animal.

mRNAs are overexpressed in cachectic animals (27-35) and patients (36-41).

Current methods for treating CACS have only a limited benefit at best. As summarized by Yavuzsen *et al* (1), examples of randomized controlled clinical trials that yielded negative, mixed or inconclusive results include trials with hydrazine sulfate, cyproheptadine, pentoxifylline, melatonin, erythropoietin with and without indomethacin, eicosapentaenoic acid, androgenic steroids, ghrelin, interferon and dronabinol. Of the drugs reviewed, only corticosteroids and progestins, demonstrated consistently positive results in the multiple randomized, and controlled clinical trials. In particular, the progesterone derivative, megestrol acetate, has been shown to increase appetite and weight (but not quality of life, survival or functional ability) in cancer cachexia patients. Evidence from clinical studies indicates that the increase in body weight observed during megestrol acetate therapy is indirect and is related to the appetite stimulation of the drug. There remains a need for better therapeutic options to help patients suffering from cachexia and anorexia. To investigate novel combination options of treating CACS based on the postulated molecular pathways, we selected two classes of existing drugs with

specific molecular action profiles: macrolide antibiotics and  $\beta$ -2 adrenergic agonists.

Illustrating the link between inflammation and CACS, Piffar *et al* (42) showed in a rat model of cancer cachexia that the non-steroidal anti-inflammatory drug naproxen reduced tumor growth and increased muscle mass, and that these beneficial effects were enhanced by the co-administration of the  $\beta$ -2 adrenergic agonist, clenbuterol. Macrolide antibiotics, structurally related to erythromycin, are also known to decrease the levels of pro-inflammatory cytokines and acute phase proteins (44-50). Specifically, roxithromycin bears apparent anti-inflammatory effects and has the ability to reduce the circulating levels of pro-inflammatory cytokines (51-55). The roxithromycin mechanism of action still remains the subject of investigation, but suppressing nuclear transcription factor  $\kappa$ B (NF $\kappa$ B) activity is an important factor (56,57).

The  $\beta$ -2 adrenergic agonists exhibit anti-catabolic in addition to the main line bronchodilator activity (34,58-62). The  $\beta$ -2 agonists have recently been demonstrated (63) to increase lean body mass by increasing protein synthesis and by interfering with the ATP-dependent ubiquitin-proteasome pathway (notably, the two mechanisms underlying muscle

Group	Inoculum	Dose (mg/kg) <sup>a</sup>		N <sup>b</sup>	Heart <sup>c</sup>		Gastrocnemius <sup>c</sup>		Carcass <sup>c,d</sup>	
		F	R		Mean	SEM	Mean	SEM	Mean	SEM
1	AH	0	0	7	0.320	0.008	0.503	0.005	93.96	0.70
2	Saline	0	0	7	0.359	0.010	0.588	0.007	96.26	0.75
3	AH	1	0	7	0.337	0.009	0.551	0.012	95.35	0.86
4	AH	0	5	9	0.320	0.009	0.493	0.004	94.35	0.71

<sup>a</sup>F, formoterol fumarate and R, roxithromycin. <sup>b</sup>The number of evaluated animals. Animals that accumulated <1 ml of ascites were not considered in the analysis. <sup>c</sup>Sample weights (g) normalized to 100 g of Day 5 total body weight. <sup>d</sup>Carcass weights after removal of the heart, gastrocnemius and ascites.

Table III. Statistical comparisons for Experiment 1.

Comparison (Group vs. Group)		Heart	Gastrocnemius	Carcass <sup>a</sup>
1	2	ns	p<0.001	ns
1	3	ns	p<0.001	ns
1	4	ns	ns	ns
2	3	ns	p<0.05	ns
2	4	p<0.05	p<0.001	ns
3	4	ns	p<0.001	ns

<sup>a</sup>Carcass weights after removal of the heart, gastrocnemius and ascites; ns, not significant.

loss in cachexia, *vide supra*). Of the approved  $\beta$ -2 adrenergic agonists, formoterol is known to be highly potent, selective and long-acting.

Accordingly, we have undertaken to study these drugs individually and in combination for the potential synergistic effect in antagonizing cachexia. We have chosen the rat ascites hepatoma model (30-34) as the system to probe the effects of the drugs and their combinations. In rats inoculated with Yoshida AH130 ascites hepatoma (AH) cells, the onset of severe cachexia is very rapid, and  $\beta$ -2 agonists (including formoterol) have previously been shown to antagonize the developed syndrome.

We therefore examined the effects of formoterol and roxithromycin, given individually and in combination, on the hallmarks of cachexia in the rat AH model. The objectives of the investigation were to: 1) reproduce the reported effects of formoterol in the rat AH model, 2) assess the anti-cachectic activity of roxithromycin and 3) probe the potential for synergy when roxithromycin and formoterol are co-administered.

## Materials and methods

**Animals.** The female Wistar rats (Charles River) were 5-6 weeks old, with a body weight of 150-225 g, on day zero (D0) of the study. The animals were fed *ad libitum* water (reverse

osmosis, 1 ppm Cl) and a NIH 31 Modified and Irradiated Lab Diet<sup>®</sup> consisting of 18.0% crude protein, 5.0% crude fat and 5.0% crude fiber. The rats were housed on irradiated Alpha-dri<sup>®</sup> bed-o-cobs<sup>®</sup> laboratory animal bedding in static micro-isolators on a 12-h light cycle at 21-22°C (70-72°F) and 40-60% humidity. This study specifically complied with the recommendations of the guide for the care and use of laboratory animals with respect to restraint, husbandry, surgical procedures, feed and fluid regulation and veterinary care.

**Tumor/saline inoculation.** Yoshida AH130 rat ascites hepatoma cells (National Cancer Institute Division of Cancer Treatment and Diagnosis, Catalog page 510, 2003) were propagated *in vivo* using female Wistar rats. The AH cells used for implantation were harvested from the donor animal ascites fluid during the log phase growth and re-suspended in phosphate-buffered saline at  $2 \times 10^7$  cells/ml. On D0, the animals in the test groups each received either an intra-peritoneal (i.p.) injection of 1.0 ml of the cell suspension (inoculum, AH) or sterile saline solution (inoculum, saline).

**Test articles.** Formoterol fumarate dihydrate (CAS 183814-30-4) was supplied by Taizhou Candorly, Beijing, China. Roxithromycin (CAS 80214-83-1) was supplied by Zhejiang Zhenyuan, Shaoxing, China. Formoterol fumarate (0.2 mg/ml) and roxithromycin (2.0 mg/ml) were formulated in 20 mM citrate, pH 5.5 and 4.5% mannitol in water using USP/NF grade materials. The formoterol and roxithromycin solutions were stored at 4°C.

**Treatment regimen.** All of the test and control articles were administered once daily for four days, starting on D1 with formoterol (nominal 1 ml per rat, adjusted for actual D0 body weight) and roxithromycin (nominal 0.5 ml) solutions and matching vehicle control solutions by i.p. injection.

**Measurements.** The total body weight was recorded for each animal on D1-D5. After animals were euthanized on Day 5, the heart and the two gastrocnemius muscles were excised and individually weighed, and the carcass was re-weighed. Then, all of the fluid was withdrawn from the peritoneal cavity, and the residual carcass weight was recorded again. Throughout, the 'carcass' weight refers to the residual carcass

Table IV. The gravimetric data for rat AH Experiment 2.

Group	Inoculum	Dose (mg/kg) <sup>a</sup>		N	Heart <sup>b</sup>		Gastrocnemius <sup>b</sup>		Carcass <sup>b,c</sup>	
		F	R		Mean	SEM	Mean	SEM	Mean	SEM
5	AH <sup>a</sup>	0	0	10	0.349	0.0060	0.487	0.0090	92.5	0.91
6	Saline	0	0	10	0.398	0.0080	0.565	0.0100	98.2	0.27
7	AH <sup>a</sup>	1	0	10	0.348	0.0090	0.499	0.0050	89.7	0.53
8	AH <sup>a</sup>	1	5	10	0.358	0.0100	0.514	0.0090	90.7	0.83
9	Saline	0	5	10	0.390	0.0110	0.575	0.0070	97.6	0.48
10	AH <sup>a</sup>	1	0	7	0.410	0.0100	0.613	0.0080	97.6	0.26

<sup>a</sup>F, formoterol fumarate and R, roxithromycin. <sup>b</sup>Sample weight normalized to 100 g of Day 5 total body weight. <sup>c</sup>Carcass weights after removal of the heart, gastrocnemius and ascites.

after removal of the heart, gastrocnemius and ascites. Tissue weights were determined on a calibrated analytical balance (readable and repeatable to 0.1 mg).

**Data analyses.** After all of the weights were recorded, the weights of the heart, each gastrocnemius muscle and residual carcass, were normalized to a Day 5 body weight of 100 g. i.e. they were expressed as fractions of the total body weight on Day 5. In some of the analyses, the mean gastrocnemius weights in the test groups within an Experiment were normalized to the mean gastrocnemius weight of the saline-inoculated, vehicle-treated group within the same Experiment. This normalization permitted the comparison of treatment groups between Experiments. The significance of differences among the means of the normalized tissue or body weight values for the various treatment groups was determined by analysis of variance (ANOVA). When the means differed significantly, Tukey's multiple comparison test was employed to determine whether any group mean differed significantly from another. The two-tailed statistical analyses were conducted at a significance level of  $p=0.05$ , utilizing the Prism 3.03 (GraphPad) for Windows. For the AH-inoculated groups (in Experiment 1 only), when an animal indicated <1.0 ml of ascites fluid, engraftment failure was assumed and such failures were excluded from statistical analysis.

## Results

**Study design.** In total, four experiments were undertaken to determine the effects of various treatment options. Table I shows the study design and drug dosing levels for the four experiments. In each Experiment, three animal groups served as controls, namely the saline-inoculated/vehicle-treated, AH-inoculated/vehicle-treated and AH-inoculated/Formoterol (1 mg/kg)-treated groups. The treatment groups have been designated using the following conventions:

Treatment group = Inoculation type/vehicle, or  
Treatment group = Inoculation type/F(0)R(0)

where, Inoculation type is either AH or saline, F is formoterol fumarate, R is roxithromycin and the parenthetical values are formoterol fumarate and roxithromycin doses (i.p. in mg/kg).


Table V. Statistical comparisons for Experiment 2.

Comparison (Group vs. Group)		Heart	Gastrocnemius	Carcass <sup>a</sup>
5	6	$p<0.05$	$p<0.001$	$p<0.001$
7	6	$p<0.05$	$p<0.001$	$p<0.001$
8	6	ns	$p<0.05$	$p<0.001$
9	6	ns	ns	ns
10	6	ns	ns	ns
5	7	ns	ns	ns
5	8	ns	ns	ns
7	8	ns	ns	ns

<sup>a</sup>Carcass weights after removal of the heart, gastrocnemius and ascites; ns, not significant.

**Experiment 1.** Table II shows the data for Experiment 1 and Table III shows the results of the ANOVA analysis. In Experiment 1, gastrocnemius muscle loss was observed to be significantly greater for the AH/vehicle group than for the saline/vehicle group (1 vs. 2), confirming the highly cachectic nature of the AH model. Animals in the AH/ F(1)R(0) group lost significantly less gastrocnemius muscle than animals in the AH/vehicle group (3 vs. 1). Animals in the AH/F(0)R(5) group lost approximately the same amount of gastrocnemius muscle as those in the AH/vehicle (4 vs. 1). The trends documented for gastrocnemius muscle weight changes in the various treatment groups roughly paralleled those observed for the heart and carcass weights (see Table II), indicating that the effects of the AH inoculation and treatments were general and not limited to the gastrocnemius muscle.

**Experiment 2.** Table IV shows data for Experiment 2 and Table V shows the results of the ANOVA analysis. In Experiment 2, the gastrocnemius weights for animals in the AH/F(1)R(5) group were not statistically significantly different from the muscle weights for animals in the AH/F(1)R(0) group (8 vs. 7). Rats in the saline/F(0)R(5) group

 SPANDIDOS PUBLICATIONS The gravimetric data for rat AH Experiment 3.

Group	Inoculum	Dose (mg/kg) <sup>a</sup>		N	Heart <sup>b</sup>		Gastrocnemius <sup>b</sup>		Carcass <sup>b,c</sup>	
		F	R		Mean	SEM	Mean	SEM	Mean	SEM
11	AH	0	0	10	0.341	0.0070	0.431	0.0040	93.6	0.52
12	Saline	0	0	10	0.417	0.0120	0.536	0.0060	95.2	0.83
13	AH	1	0	10	0.376	0.0140	0.489	0.0090	93.1	0.69
14	AH	1	40	10	0.350	0.0360	0.528	0.0100	95.9	0.58
15	Saline	0	40	10	0.400	0.0070	0.529	0.0080	96.5	0.52

<sup>a</sup>F, formoterol fumarate and R, roxithromycin. <sup>b</sup>Sample weights normalized to 100 g of Day 5 total body weight. <sup>c</sup>Carcass weights after removal of the heart, gastrocnemius and ascites.

Table VII. Statistical comparisons for Experiment 3.

Comparison (Group vs. Group)		Heart	Gastrocnemius	Carcass <sup>a</sup>
11	12	p<0.001	p<0.001	ns
11	13	ns	p<0.001	ns
11	14	ns	p<0.001	ns
11	15	not compared	not compared	not compared
12	13	ns	p<0.05	ns
12	14	ns	ns	ns
12	15	ns	ns	ns
13	14	ns	p<0.05	p<0.05
13	15	not compared	not compared	not compared
14	15	not compared	not compared	not compared

<sup>a</sup>Carcass weights after removal of the heart, gastrocnemius and ascites; ns, not significant.

Table VIII. Gravimetric data for rat AH Experiment 4<sup>d</sup>.

Group	Inoculum	Dose (mg/kg) <sup>a</sup>		Heart <sup>b</sup>		Gastrocnemius		Carcass <sup>b,c</sup>	
		F	R	Mean	SEM	Mean	SEM	Mean	SEM
16	AH <sup>a</sup>	0	0	0.323	0.0080	0.453	0.0051	90.6	0.80
17	Saline	0	0	0.405	0.0069	0.556	0.0080	96.5	0.76
18	AH <sup>a</sup>	1	0	0.367	0.0160	0.502	0.0120	92.2	0.70
19	AH <sup>a</sup>	1	25	0.344	0.0085	0.477	0.0080	91.4	0.64
20	AH <sup>a</sup>	1	50	0.373	0.0106	0.520	0.0097	94.8	0.72
21	AH <sup>a</sup>	0	50	0.338	0.0130	0.500	0.0092	94.1	0.61

<sup>a</sup>F, formoterol fumarate and R, roxithromycin. <sup>b</sup>Sample weight normalized to 100 g of Day 5 total body weight. <sup>c</sup>Carcass weights after removal of the heart, gastrocnemius and ascites. <sup>d</sup>N=10 animals per group. <sup>e</sup>N=6 animals per group.

had approximately the same gastrocnemius muscle weights as rats in the saline/vehicle group (9 vs 6). Animals in the saline/F(1)R(0) group had higher (but not statistically signifi-

cantly different) gastrocnemius muscle weights than animals in the saline/vehicle group (10 vs. 6), consistent with the known (30) anti-catabolic effects of formoterol fumarate in rats.



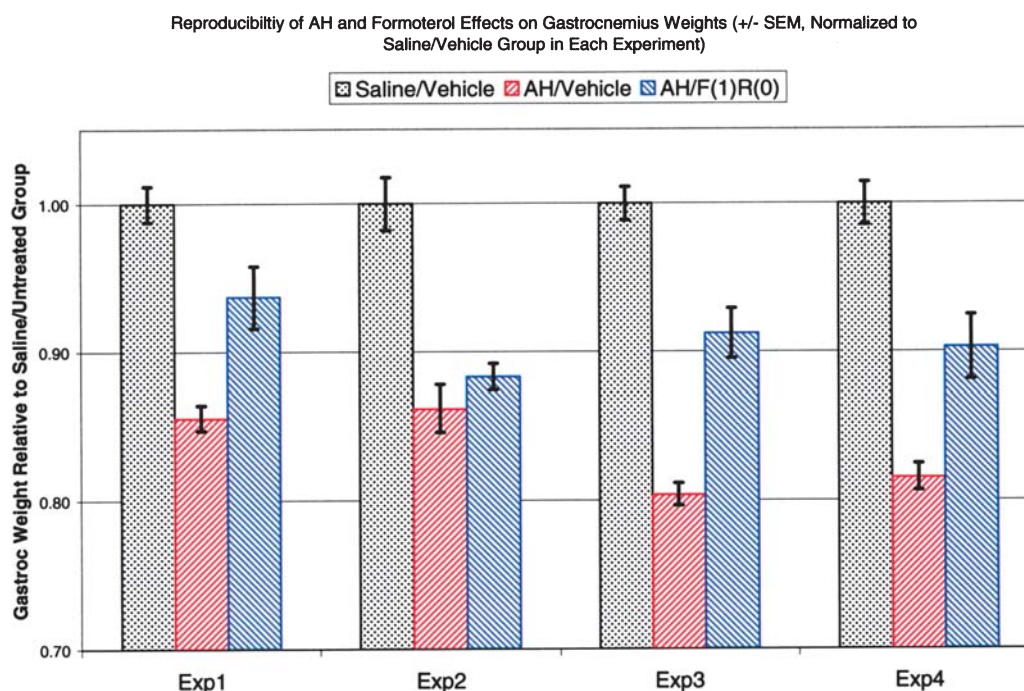


Figure 1. Reproducibility of the formoterol effect on gastrocnemius muscle weights in Experiments 1-4.

Table IX. Statistical comparisons for Experiment 4.

Comparison (Group vs. Group)		Significance for tissue		
		Heart	Gastrocnemius	Carcass <sup>a</sup>
16	17	p<0.05	p<0.05	p<0.05
16	18	ns	p<0.05	ns
16	19	ns	ns	ns
16	20	p<0.05	p<0.05	p<0.05
16	21	ns	p<0.05	p<0.05
17	18	ns	p<0.05	p<0.05
17	19	p<0.05	p<0.05	p<0.05
17	20	ns	ns	ns
17	21	p<0.05	p<0.05	ns
18	19	ns	ns	ns
18	20	ns	ns	ns
18	21	ns	ns	ns
19	20	ns	p<0.05	p<0.05
19	21	ns	ns	ns
20	21	ns	ns	ns

<sup>a</sup>Carcass weights after removal of the heart, gastrocnemius and ascites; ns, not significant.

*Experiment 3.* Table VI shows the data for Experiment 3 and Table VII shows the results of the ANOVA analysis. In Experiment 3, rats in the AH/F(1)R(40) group had much greater gastrocnemius muscle weights than rats in the

AH/F(1)R(0) group (14 vs. 13). The gastrocnemius muscle weight differences between Groups 14 and 13 were found to be statistically significant. For animals in the AH/ F(1)R(40) group, the gastrocnemius weights were not statistically significantly different from the muscle weights for animals in the saline/vehicle control group (14 vs. 12). Gastrocnemius muscle weights were also not statistically significantly different in the saline/F(0)R(40) group compared with the saline/vehicle control group (15 vs. 12).

*Experiment 4.* Table VIII shows the data for Experiment 4 and Table IX shows the results of the ANOVA analysis. In Experiment 4, the gastrocnemius net muscle weights for rats in the AH/F(1)R(25) group were not statistically significantly different from those for rats in the AH/F(1)R(0) group (19 vs. 18). For animals in the AH/F(1)R(50) group, gastrocnemius weights were higher than those for animals in the AH/F(1)R(0) group (20 vs. 18), but the differences were not statistically significant. The animals in the AH/F(0)R(50) group demonstrated significantly higher gastrocnemius weights than animals in the AH/vehicle group (21 vs. 16). For the rats in the AH/F(0)R(50) group, gastrocnemius weights were essentially identical to those for rats in the AH/F(1)R(0) group and slightly (but not statistically significantly) lower than gastrocnemius for rats in the AH/F(1)R(50) group (21 vs. 18 and 21 vs. 20).

## Discussion

In the four Experiments, the observed effects of AH-inoculation (with or without various treatments) on the heart and carcass weights were generally parallel to the effects on gastrocnemius muscle weights. AH-inoculation and treatment effects on the body and tissue weights are, therefore, general and not limited to the gastrocnemius muscle. Experimental uncertainty was

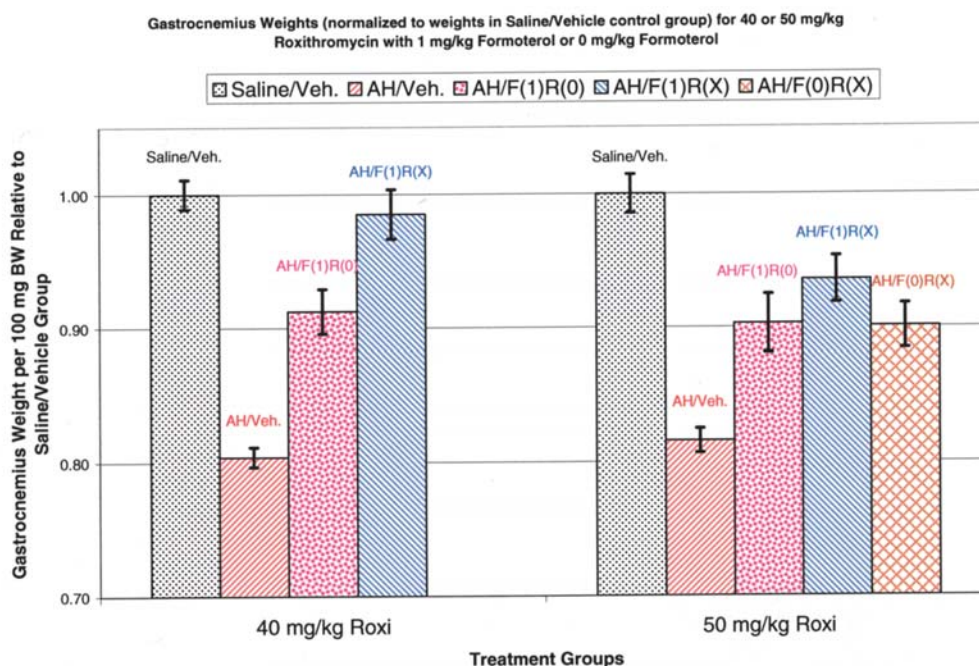


Figure 2. Gastrocnemius weights (mean ± SEM) for 40 or 50 mg/kg roxithromycin in AH-inoculated rats.

Table X. Comparison of the gastrocnemius data with results reported by Busquets *et al* (30).

Inoculum/treatment <sup>a</sup>	Difference in mean gastrocnemius weights (g per 100 g of Day 5 body weight)				
	Busquets	Experiment 1	Experiment 2	Experiment 3	Experiment 4
AH/Vehicle - saline/vehicle	-0.088	-0.086	-0.078	-0.105	-0.102
AH/F(1)R(0) - AH/vehicle	0.054	0.048	0.012	0.058	0.048
AH/F(1)R(0) - saline/vehicle	-0.038	-0.038	-0.066	-0.047	-0.054

<sup>a</sup>Formoterol dose = 1 mg/kg i.p.

Table XI. The statistical significance of the difference in gastrocnemius weights in AH-inoculated rats receiving various treatments compared with gastrocnemius weights in AH/vehicle controls and in AH/F(1)R(0) controls.<sup>a</sup>

Difference in weights Formoterol (mg/kg) vs. roxithromycin (mg)	AH/treatment vs. AH/vehicle F(0)	AH/treatment vs. AH/vehicle F(1)	AH/treatment vs. AH/F(1)R(0) F(0)	AH/treatment vs. AH/F(1)R(0) F(1)
R (5)	ns	ns	ns	ns
R (25)	not tested	ns	not tested	ns
R (40)	not tested	p<0.001	not tested	p<0.05
R (50)	p<0.01	p<0.001	ns	ns

<sup>a</sup>ns, not significant at p=0.05.

lower for measurements of the gastrocnemius weight than for the heart and carcass weights. Therefore, the following sections focus on gastrocnemius data.

Fig. 1 demonstrates the reproducibility of the rat AH model with respect to gastrocnemius muscle weights (normalized to the gastrocnemius weights for the saline/vehicle groups in each Experiment). For the four Experiments, gastrocnemius

muscle loss in AH/vehicle groups was measured as ~15-20% compared to the muscle weight in the saline/vehicle groups at Day 5. In the AH/F(1)R(0) groups, gastrocnemius muscle loss was measured as only ~10% of the muscle mass in the saline/vehicle groups. The variability in response to AH-inoculation with either the vehicle or 1 mg/kg formoterol fumarate treatment probably reflects the highly aggressive nature of

the model and the fact that there is a very steep drop in muscle mass over the relatively short (5 day) course of each Experiment.

It is therefore useful to compare the gastrocnemius muscle data from Experiments 1-4 with the data reported by Busquets *et al* (30) for the same rat AH model.

Table X shows that the changes in the gastrocnemius weights among the groups from Experiments 1-4 are identical in direction (positive or negative differences) and very similar in magnitude to the data reported by Busquets *et al* (30), thereby confirming the general conclusions of this report.

In the saline-inoculated rats, none of the investigated treatments demonstrated statistically significant effects on the gastrocnemius weights. Animals in the saline/F(1)R(0) group (Group 10, Experiment 2), had greater gastrocnemius muscle weights than those in the saline/vehicle control group (Group 6), consistent with literature reports (15) on the anti-catabolic activity of formoterol.

In the AH-inoculated rats, the treatment group effects on gastrocnemius weights can be compared to the AH/vehicle control group and to the AH/F(1)R(0) control groups.

Table XI is a grid of the treatment groups (0 mg/kg formoterol fumarate versus 5, 25, 40 or 50 mg/kg roxithromycin and 1 mg/kg formoterol fumarate versus 5, 25, 40 or 50 mg/kg roxithromycin). The table shows the degree to which the gastrocnemius weight differences between the treatment and control (either AH/vehicle or AH/F(1)R(0)) groups achieved statistical significance.

With respect to the gastrocnemius data in AH-inoculated rats, the treatment groups receiving 5 or 25 mg/kg roxithromycin (either alone or in combination with 1 mg/kg formoterol fumarate) did not differ significantly from the control groups. The AH/F(1)R(40) treatment group was statistically significantly different from either the AH/vehicle or the AH/F(1)R(0) control group. The AH/F(0)R(50) treatment group was statistically significantly different from the AH/vehicle but essentially identical to the AH/F(1)R(0) control group. The AH/F(1)R(50) treatment group was statistically significantly different compared with the AH/vehicle control group but not with the AH/F(1)R(0) control group, although the AH/F(1)R(50) group had a somewhat higher gastrocnemius muscle mass than the AH/F(1)R(0) control group.

Fig. 2 shows that the combination of 40 mg/kg roxithromycin with 1 mg/kg formoterol fumarate prevented muscle loss significantly better than did 1 mg/kg formoterol alone. Similarly, the muscle loss was lower for the combination of 50 mg/kg roxithromycin plus 1 mg/kg formoterol fumarate than for either 50 mg/kg roxithromycin or 1 mg/kg formoterol fumarate administered individually. The differences in the gastrocnemius weights between the AH/F(1)R(50) group and either the AH/F(1)R(0) or the AH/F(0)R(50) group, however, did not achieve statistical significance.

In conclusion, herein we reported a novel observation that roxithromycin exerts anti-cachectic effects in the rat AH model. Roxithromycin administered at 50 mg/kg alone was just as effective as 1 mg/kg formoterol fumarate in preventing AH-induced gastrocnemius muscle loss. Although the data in this report cannot conclusively establish any mechanism(s) for the roxithromycin effect on cachexia, the suppression of pro-inflammatory cytokines is the most likely explanation (42-50).

The anti-cachectic activity of formoterol fumarate shown here is consistent with literature reports (30-34) and with a mechanism involving the formoterol fumarate inhibition of proteolysis via the ubiquitin-proteasome pathway. The data reported here suggest that roxithromycin and formoterol fumarate work via independent mechanisms to antagonize cachexia in the AH rat model. It could, therefore, be concluded that the co-administration of formoterol fumarate and roxithromycin may offer the promise of an effective, multi-modal treatment approach to the treatment of CACS.

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