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Research Article

A Combination of Formoterol and the Histone Deacetylase Inhibitor AR42 has No Effects on Muscle Mass in Tumor-Bearing Rats

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ABSTRACT

Background: Accelerated muscle and adipose tissue loss are two of the main aspects of cancer cachexia. β2-agonists seem to be successful in the treatment of cachexia in experimental animals. The aim if the present investigation was to study the effects on body weight loss in tumor-bearing animals of a combination of formoterol and AR-42, an inhibitor of histone deacetylase (HDAC).

Methods: Rats were divided into two groups, namely controls (C) and tumor-bearing (T). TB group was further divided into four subgroups: untreated (saline as a vehicle), treated with Formoterol (F) (0,3 mg/kg body weight in saline, subcutaneous (s.c.), daily), treated with AR-42 (A) (20 mg/kg body weight in olive oil, intragastric (i.g.), only the last 4 days). and double-treated treated (TFA) with Formoterol (0,3 mg/kg body weight, subcutaneous (s.c.), daily) and AR-42 (20 mg/kg body weight in olive oil, intragastric (i.g.), only the last 4 days). 7 days after tumor transplantation, muscle weights, grip force and total physical activity were determined in all experimental groups.

Results: The presence of the Yoshida AH-130 ascites hepatoma induced severe muscle wasting in rats. Treatment of the tumor-bearing animals with the beta2-agonist formoterol (0,3 mg/kg), resulted in a significant improvement in the cachectic state of the animals. Treatment of the tumor-bearing animals with AR42 did not result in any effects on muscle wasting in the cachectic rats. Furthermore, the combination of formoterol and AR42 showed no additional effects to those observed with just formoterol.

Conclusion: The results presented question the previously described effects of AR42 on cancer cachexia, probably due to its effect on tumor growth.

Background

A consensus group defined cachexia as a "complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass. The prominent clinical feature of cachexia is weight loss in adults (corrected for fluid retention) or growth failure in children (excluding endocrine disorders). Anorexia, inflammation, insulin resistance and increased muscle protein breakdown are frequently associated with cachexia. Cachexia is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption and hyperthyroidism and is associated with increased morbidity" [1]. From 50 t0 80% of cancer patients experiment the cachexia syndrome. In fact, cachexia is a useful tool for survival prediction, being held responsible for more than 20% of the deaths of cancer patients [2]. It is directly responsible for a reduction in physical activity and quality of life and decreases the efficacy and outcome of anticancer therapy [3-5]. Both adipose tissue and muscle weights are reduced during cancer cachexia; however, muscle wasting is the main event. In fact, the loss of body weight and muscle mass are directly involved not only with survival but also the physical performance of the patient [6].

Many therapeutic approaches and strategies have been described to treat the cachexia syndrome, but, unfortunately, none of them are able to totally reverse the weight loss. Basically, the different targets addressed

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in the treatment of the syndrome are counteracting anorexia and/or neutralizing metabolic disturbances [7, 8]. Concerning the neutralization of the metabolic alterations, β2-agonists, formoterol in particular, had important anti-cachectic effects [9]. The mode of action of this drug is based on its ability to prevent muscle wasting by inhibiting proteolysis and apoptosis in skeletal muscle. Thereby, formoterol decreased the activation of the ubiquitin-dependent proteolytic system, the main mechanism activated in muscle wasting conditions, as well as decreased muscle apoptosis in tumor-bearing animals [9, 10]. The anti-wasting effects of the drug were also observed in terms of total physical activity and grip force, thus resulting in an improvement in physical performance in cachectic tumor-bearing rats [11]. In humans, the combination of formoterol and the orexigenic drug megestrol acetate also resulted in a promising therapy in cancer cachexia [12].

Histone deacetylases (HDAC) regulate gene transcription through the elimination of acetyl groups presents in lysine, increasing the positive charge and consequently its affinity for DNA (which is negatively charged) [13]. Modification of histones by acetylation plays a key role in epigenetic regulation of gene expression and is controlled by the balance between histone deacetylases (HDAC) and histone acetyltransferases (HAT). HDAC inhibitors induce cancer cell cycle arrest, differentiation and cell death, reduce angiogenesis and modulate immune response [13]. In fact, AR-42, an inhibitor of HDAC, has been proved to have antitumoral effects in both hematologic and solid tumor malignancies, and this effect has been investigated in clinical trials for the treatment of patients with lymphoma, multiple myeloma and acute myelogenous leukemia [14-25].

Additionally, another function for HDAC has been described: it can modify the acetylation degree of non-histone proteins, such as transcription factors. This is the function that seems to be interesting for treating cancer cachexia. Inhibiting HDAC through AR42 administration could be a good therapeutic tool to stop muscle wasting, because acetylation of transcription factor FoxO, which is involved in the ubiquitin-dependent proteolytic system that includes genes such as MuRF-1 and atrogin-1, could stop muscle wasting programme [26]. A recent study shows how AR-42 treatment allows the recovery of basal expression of those genes and therefore, it stops the symptoms associated with cancer cachexia [27]. Other HDAC inhibitors (such as MS-275) prevent contractile dysfunction during skeletal muscle disuse and reduces the extent of fiber atrophy [28]. Another potential effect of HDAC inhibitors is that these compounds could behave as exercise mimicking agents (a well-known strategy against cachexia) [29]. Bearing all this in mind, the aim of the present investigation was to explore if the combination of formoterol and AR42 has any synergistic effect on cancer-related cachexia.

Materials and Methods

I Animals

6 weeks old male Wistar rats (Harlan, Barcelona, Spain) were housed in individual cages and maintained at a constant temperature of 22 ± 2 °C with a regular light-dark cycle (light from 08:00 a.m. to 08:00 p.m.) and free access to food and water. Experimental cachexia was obtained through i.p. injection of 100×10^6 AH-130 Yoshida ascites hepatoma cells obtained from exponential tumors as described previously [30]. Food intake was measured daily. The experimental protocol was approved by the Ethical Committee of the University of Barcelona and all animal manipulations were made in accordance with the European Community guidelines for the use of laboratory animals [31].

II Experimental Design

Rats were divided in two groups, namely controls (C) and tumor-bearing (TB). T group was further divided into four subgroups: untreated (T) (saline/olive oil as a vehicle), treated with formoterol (F) (0.3 mg/kg body weight in saline, subcutaneous (s.c.), daily), treated with AR42 (A) (20 mg/kg body weight in olive oil, intragastric (i.g.), only the last 4 days), and double-treated (F+A) treated with formoterol (0,3 mg/kg body weight, subcutaneous (s.c.), daily) and AR42 (20 mg/kg body weight in olive oil, intragastric (i.g.), only the last 4 days). Seven days after tumor transplantation, animals were weighted and anaesthetized with an intraperitoneal (i.p.) injection of ketamine/xylazine mixture (3:1) (Imalgene® and Rompun® respectively). Tumor volume and total cell number were assessed at the day of sacrifice. Tissues were rapidly excised, weighted, and frozen in liquid nitrogen.

III Biochemicals

Formoterol was kindly provided by Industriale Chimica s.r.l. (Saronno, Italy), AR42 ((S)-(+)-N-hydroxy-4-(3-methyl-2-phenyl-butyrylamino) benzamide was obtained from Arno Therapeutics (Flemington, New Jersey) [32].

IV Grip Force Assessment

Skeletal muscular strength in rats was quantified by the grip-strength test [33]. The grip-strength device (Panlab-Harvard Apparatus, Spain) comprised a pull bar connected to an isometric force transducer (dynamometer). Basically, the grip strength meter was positioned horizontally, and the rats are held by the tail and lowered towards the device. The animals were allowed to grasp the bar and were then pulled backwards in the horizontal plane. The force applied to the bar just before it lost grip was recorded as the peak tension. At least three measurements were taken per rat and the results were averaged for analysis. The data are presented as g/g initial body weight.

V Statistical Analysis

Average (arithmetic mean) and standard error of the mean (SEM) were calculated for each studied variable. Statistical analysis of the data was performed by means of the Student's t-test.

Results

As can be seen in (Table 1), tumor-bearing animals experimented important decreases in body weight, carcass weight and food intake. The gastrointestinal tract was also reduced by the presence of the tumor. As previously seen in other publications in our group, formoterol treatment significantly improved these parameters (Table 1) [34, 35]. However, no effect of AR-42 treatment was observed neither on body weight nor carcass. In fact, food intake was significantly decreased by the treatment

as compared with the untreated tumor-bearing animals. The combination of formoterol and AR-42 had no effects on the above-mentioned parameters as compared with the animals treated with the inhibitor of HDAC.

Table 1: Effects of formoterol and AR42 treatments on food intake, body weight and tumor content in tumor-bearing rats.

Parameters	**Experimental groups**				
	C	**T**	**T+F**	**T+A**	**T+F+A**
IBW	176 ± 5	178 ± 3	180 ± 2	176 ± 11	177 ± 4
FBW	216 ± 6	182 ± 6 ##	189 ± 6	172 ± 6	174 ± 4
ΔBW	40 ± 4	4 ± 5 ###	10 ± 5	-5 ± 5	-2,4 ± 4
Food intake	71 ± 2	63 ± 3 #	69 ± 1	51 ± 4 *	55 ± 5
Carcass	87 ± 3	78 ± 1 ##	80 ± 1	76 ± 2	75 ± 2
GIT	10527 ± 453	6756 ± 316 ###	6480 ± 296	8043 ± 699	7546 ± 417
Tumor					
Volume (mL)	-	41 ± 1	46 ± 4	40 ± 3	39 ± 4
Total cell number (10^6)	-	5337 ± 539	4533 ± 386	3289 ± 458 *	3438 ± 482 *

Results are mean ± SEM for the number of animals: C (6), T (7), T+F (5), T+A (4), T+F+A (7). IBW: Initial Body Weight and FBW: Final Body Weight are expressed in g. Food intake is expressed as g/100g IBW and refers to the cumulative intake (7 days). Carcass is expressed in g/100 g IBW. GIT: Gastrointestinal Tract is expressed in mg/100 g IBW. Values that are significantly different by the Student's t-test from the control group (C) are indicated by # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, and from the tumor non-treated animal group (T) are indicated by * $p < 0.05$.

C: rats without tumor; T: Tumor-bearing rats; T+F: Treated with Formoterol; T+A: Treated with AR42; T+F+A: Treated with both Formoterol and AR42.

Table 2: Effects of formoterol and AR42 treatments on muscles and adipose tissue weights in tumor-bearing rats.

Parameters	**Experimental groups**				
	C	**T**	**T+F**	**T+A**	**T+F+A**
Muscle Weights					
GSN	671 ± 9	568 ± 16 ###	650 ± 11 ***	569 ± 14	602 ± 13
Tibialis	216 ± 3	188 ± 5 ###	212 ± 4 ***	179 ± 7	190 ± 6
Soleus	48 ± 1	44 ± 1 #	47 ± 1 *	45 ± 2	42 ± 1
EDL	52 ± 2	44 ±1 ###	49 ± 1 ***	42 ± 1	45 ± 1
Heart	401 ± 14	348 ± 9 ##	345 ± 18	342 ± 9	353 ± 31

Results are mean ± SEM for the number of animals: C (12), T (13), T+F (13), T+A (4), T+F+A (7). Values that are significantly different by the Student's t-test from the control group (C) are indicated by # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, and from the tumor non-treated animals group (T) are indicated by * $p < 0.05$, *** $p < 0.001$.

GSN: Gastrocnemius muscle; EDL: Extensor Digitorum Longus; C: rats without tumor; T: Tumor-bearing rats; T+F: Treated with Formoterol; T+A: Treated with AR42; T+F+A: Treated with both Formoterol and AR42.

Table 2 clearly shows that tumor-bearing animals suffered an important decrease in the mass of all the individual skeletal muscles studied. Formoterol treatment significantly improved muscle weight. However, treatment with AR-42 did not show any benefits on muscle weight. On the same lines, the combination of formoterol and AR-42 showed no synergistic effects but, in fact, the positive effects alone were not seen. Since muscle mass and function are not necessarily correlated, we decided to measure muscle force in the different experimental groups. As can be seen in (Table 3), the presence of the tumor promoted a significant decrease in grip force which was improved by formoterol as previously described [35]. Treatment of the tumor-bearing animals with AR-42 resulted in no improvement of grip force as compared with tumor-bearing animals. Similarly, the combination with formoterol and AR-42 did not show any synergistic effects.

Table 3: Effects of formoterol and AR42 treatments on grip force in tumor-bearing rats.

Parameters	**Experimental groups**				
	C	**T**	**T+F**	**T+A**	**T+F+A**
Grip force day 0	340 ± 11	343 ± 12	342 ± 11	286 ± 18 *	322 ± 27
Grip force day 7	426 ± 20	357 ± 14 ##	449 ± 11 ***	257 ± 12 **	377 ± 13
Δ grip force	86 ± 19	15 ± 21 #	107 ± 11 ***	-29 ± 23	55 ± 24

Results are mean ± SEM for the number of animals: C (12), T (13), T+F (13), T+A (4), T+F+A (7). Δ grip force was calculated as [(grip force day 7- grip force day 0)/ IBW] * 100. Values that are significantly different by the Student's t-test from the control group (C) are indicated by # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, and from the tumor non-treated animal group (T) are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

C: rats without tumor; T: Tumor-bearing rats; T+F: Treated with Formoterol; T+A: Treated with AR42; T+F+A: Treated with both Formoterol and AR42.

Discussion

A recent investigation suggested an involvement of histone deacetylase (HDAC) in skeletal muscle atrophy [28]. Indeed, HDAC activates the transcription factor FoxO and seems to be a sufficient mechanism for inducing skeletal muscle atrophy [28]. Bearing this in mind, previous investigations have used AR42 (an inhibitor of HDAC) for the treatment of muscle wasting associated with cancer cachexia using both the C-26 colon adenocarcinoma and Lewis lung carcinoma (LLC) models [27]. In addition, the same inhibitor has proven its efficacy as an antitumoral agent in both cancer cell lines and tumor xenografts and transgenic mouse models of pancreatic cancers [36].

Taking all this into consideration, the object of the present investigation was: i) to examine the role of AR-42 in both, tumor growth reduction and muscle wasting in a rat cancer cachexia model and ii) to examine a combination of a proven anti-muscle wasting agent (formoterol) with AR-42 in order to analyse possible synergistic effects [34, 35]. Concerning tumor growth, while formoterol treatment did not influence, AR-42 clearly and significantly decreased total tumor cell number by 38%. Interestingly, the combination of formoterol and AR-42 also resulted in a significant decrease of the tumor (Table 1). These data agree with the previous results concerning the suppression of tumor growth of the AR42 [36]. In fact, the positive effects found in previous investigations concerning a reduction of muscle wasting by AR-42 could be a consequence of the effects of the inhibition on the tumor growth [36]. Indeed, at least in pre-clinical models, any drug decreasing tumor growth is invariably associated with an improvement of muscle wasting [37-39]. Our results do not show any benefit of the inhibition on muscle mass or function, this possibly being associated with the toxicity of the inhibitor. Although the dose used in this investigation was very similar to the ones previously investigated, toxicity leads to a decreased food intake (Table 1) together with a clear anaemia induced by AR-42 (results not shown) [27, 28, 36]. Interestingly, another investigation did not find any improvements of deacetylase inhibitors in cachexia in tumor-bearing mice despite modulation of the myostatin/follistatin axis [40].

Conclusion

In conclusion, the use of AR-42 to prevent muscle atrophy associated with cancer cachexia is questionable and additional investigations are, therefore, needed.

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Conflicts of Interest

Each author has participated sufficiently, intellectually or practically, in the work to take public responsibility for the content of the article, including the conception, design, and for data interpretation. All authors have read and approved the final manuscript. All authors of this research have not conflict of interest related with employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding: Sílvia Busquets, Marta Castillejo, Queralt Jové, Alina Noguera, Francisco J. López-Soriano and Josep M. Argilés declare that they have no conflict of interest.

Ethical Approval and Consent

The experimental protocol was approved by the Ethical Committee of the University of Barcelona and all animal manipulations were made in accordance with the European Community guidelines for the use of laboratory animals [31].

Author Contributions

MC: Food intake follow up; QJ: Isolation of individual muscles; AN: Tumor measurements; FLS: Laboratory supervisor; JMA: Direction; SB: Tumor implantation and follow up.

Abbreviation

EDL: Extensor Digitorum Longus
GSM: Gastrocnemius Muscle
HDAC: Histone Deacetylase
HAT: Histone Acetyltransferases
I.G.: Intragastric Administration
S.C.: Subcutaneous Administration
TB: Tumor Bearers
A: Animals treated with AR-42
F: Animals treated with Formoterol
F+A: Double-treated Animals

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