β-Hydroxy-β-Methylbutyrate as a Countermeasure for Cancer Cachexia: A Cellular and Molecular Rationale

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Abstract: Cancer cachexia is a life-threatening condition characterized by involuntary body weight loss and skeletal muscle wasting. In addition to being associated with poor prognosis and reduced survival, patients with cachexia exhibit a critical loss of physical function that impinges upon their ability to perform basic activities of daily living. Consequently, there is a loss of independence and a drastically reduced quality of life. Despite being a major unmet medical need of patients, very few treatment options exist. Maintaining muscle mass represents an important objective in the cancer patient trajectory not only because it relates to one's capacity to perform activities of daily living, but also because muscle preservation may be a critical determinant of survival while in a tumor-bearing state. In this regard, research has been directed towards identifying countermeasures effective in preserving muscle. With respect to nutritional approaches, administration of the leucine metabolite β-hydroxy-β-methylbutyrate (HMB) could be a viable component in multi-modal therapies targeting cancer cachexia. Evidence suggests that HMB treatment promotes regenerative events (i.e. myogenic program), suppresses protein degradation, and activates signaling pathways preceding protein synthesis and skeletal muscle growth. HMB therefore, could conceivably act on key regulatory events driving cancer cachexia, thereby favoring muscle growth/preservation. In this review, we take a mechanistic approach in making a case for the use of HMB provision as a possible therapeutic strategy for cancer cachexia by highlighting the cellular and molecular aspects of HMB function.

Keywords: HMB, atrophy, skeletal muscle, protein turnover, regeneration, signaling.

INTRODUCTION

At least half of all cancer patients undergo cachexia [1], a paraneoplastic condition characterized by profound skeletal muscle wasting and unintended weight loss with or without reductions in fat mass [2]. While most cancer patients can undergo cachexia, the incidence varies depending on the type of malignancy present [3]. In a sample of 3,000 patients, cachexia (using weight loss > 5% as the diagnostic criteria) occurred at a greatest frequency in those bearing gastric and pancreatic tumors (~85%), followed by those with malignancies of the lung (~57%), colon (~54%), and breast (~30%) [3]. Regarding patient outcomes, quality of life and prognosis are adversely affected on a number of levels. Loss of strength [4, 5], premature fatigue [6], and reduced muscle oxidative capacity [7] have been reported to occur, which in turn may compromise one's ability to perform activities of daily living. Moreover, cachexia has been associated with lower treatment efficacy [8], increased toxicity in response to chemotherapy [9], and reduced survival [10]. Overall, cancer cachexia has been reported to account for approximately 20-30% of cancer-related deaths [11, 12].

Preventing or reversing cachexia represents an important endeavor in the cancer patient trajectory because the preservation of muscle mass has been shown to have direct bearing on an organism's survival while in a tumor bearing state [13]. Unfortunately, attempts to identify effective therapies have been only marginally successful [2, 11, 12]. To address the burden of cachexia, research has been directed towards nutritional and pharmacological interventions. While the former has been shown to restore body weight losses, increased fat mass or water retention often account for this outcome [14-16]. Regarding the latter, candidate pharmacological agents are in various stages of

randomized trials including anti-tumor necrosis factor-α agents [17, 18], cyclooxygenase-2 inhibitors [19], anti-IL-6 antibodies [20], anabolic steroid analogues [21], and ghrelin mimetics [22]. Although several agents have demonstrated promise as forms of treatment, none are currently approved for use [23]. Overall, very few treatment options exist. According to Reid *et al*. [24], patients undergoing cachexia expressed frustration at both the lack of information on supportive therapies and the unresponsiveness of health care providers in recognizing and dealing with the condition. Cancer cachexia therefore represents a major unmet medical need of patients [1, 24]. Research devoted to defining effective therapies may enable patients and their family members to be better informed while also producing improved countermeasure strategies.

In this regard, β-hydroxy-β-methylbutyrate (HMB) provision could be a viable component in therapeutic strategies for cancer cachexia. HMB is produced endogenously from α-ketoisocaproate, a byproduct of leucine metabolism, and may serve as a precursor for cholesterol synthesis [25]. Because of this particular function, HMB has been suggested to have a role in the maintenance of muscle cell membrane integrity, which may be favorable in conditions of increased mechanical stress such as skeletal muscle loading (e.g. resistance exercise) [26]. Recent work has also provided evidence that HMB suppresses proteasome activity [27], promotes myogenic events [28], and functions as a signal for muscle growth by way of enhanced translation initiation signaling [29]. The apparent activation of regenerative and growth promoting pathways by HMB provides a mechanistic basis for its use as a countermeasure against catabolic stressors. In this review, we provide an overview of major regulatory events believed to have a key role in the development of cancer cachexia. We also make a case for the potential therapeutic application of HMB by providing evidence demonstrating its ability to act on several proposed mechanisms in a manner that could favor the preservation of muscle.

MECHANISMS OF CANCER CACHEXIA

Defining the etiology of cancer cachexia remains a work in progress; however, mechanistic examinations in animal models

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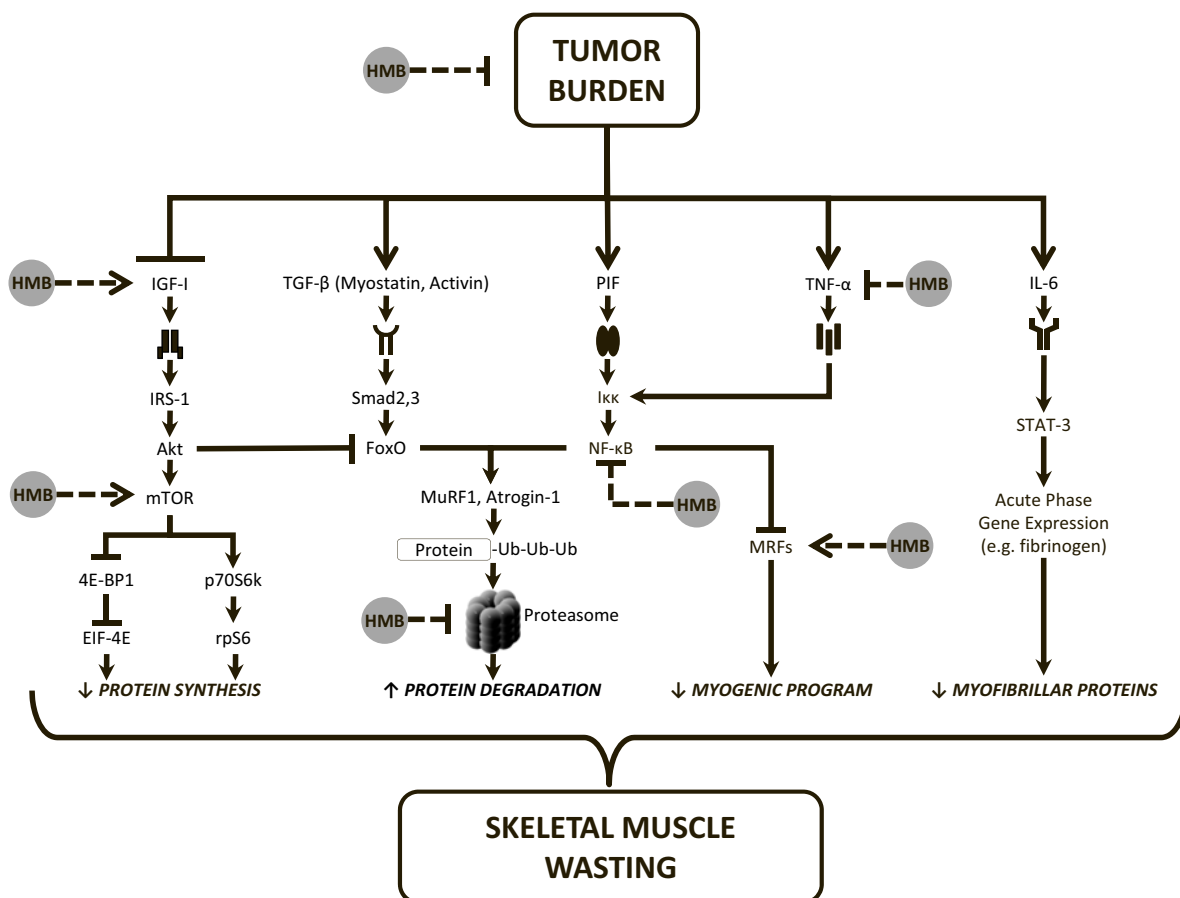#Contributed Equally

Fig. (1). Schematic representation of signaling pathways in cancer cachexia (solid lines) and potential countermeasure effects of HMB (broken lines). Arrowheads represent activation, whereas bars indicate inhibitory actions. HMB, β-hydroxy-β-methylbutyrate. IGF-I, insulin-like growth factor-I. IRS-1, insulin receptor substrate-1. Akt, protein kinase B. mTOR, mammalian target of rapamycin. EIF-4E, eukaryotic translation initiation factor-4E. 4E-BP1, EIF-4E-binding protein 1. p70S6k, 70 kDa ribosomal protein S6 kinase. rpS6, ribosomal protein S6. TGF-β, transforming growth factor-β. FoxO, forkhead box O. MuRF1, muscle ring finger 1. Ub, ubiquitin. PIF, proteolysis-inducing factor. NF-κB, nuclear factor kappa B. Iκκ, IκB kinase. MRFs, myogenic regulatory factors. TNF-α, tumor necrosis factor-α. IL-6, interleukin-6. STAT-3, signal transducer and activator of transcription-3.

have provided much insight on potential mediators and signaling pathways. It is generally accepted that the wasting of skeletal muscle central to the cachectic phenotype arises from tumor-host interactions [30]. For instance, the tumor can secrete products that act directly on skeletal muscle to signal the degradation of proteins [31, 32]. Meanwhile, the host mounts an inflammatory response to the presence of the tumor that when persistent, can promote tissue catabolism [33]. There may also be a reduction of circulating or local anabolic factors as a result of the underlying disease [34, 35]. The collective net effect of these components alters the balance of protein metabolism to favor degradation over synthesis, thereby leading to muscle atrophy. Several mediators and pathways implicated in the development of cachexia have been described below. Although they have been presented separately, it should be noted that a degree of cross-talk exists between the pathways (Fig. (**1**)).

Interleukin-6 Signaling

Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and interferon-γ (IFN-γ) appear to be key mediators of cachexia. IL-6 in particular appears to be a strongly associated with cachexia based on evidence from animals and humans. For instance, elevated levels of circulating IL-6 are frequently observed in the widely employed C26 [13, 36], lewis lung [37], and Apc^Min/+ [37-39] murine models of cancer cachexia. Likewise, increased circulating IL-6 has also

been reported in cancer patients demonstrating cachexia [40, 41]. Recently, compelling evidence has been documented to substantiate the role of IL-6 as a critical contributor to cachexia in the Apc^Min/+ mouse model of colon cancer. Baltgalvis and colleagues [38] tested the effects of IL-6 ablation by comparing Apc^Min/+/IL-6^-/- mice (IL-6 null) to Apc^Min/+ and C57BL/6 mice. Muscle weight in the Apc^Min/+ mice was significantly lower than both C57BL/6 and Apc^Min/+ /IL-6^-/- mice. In fact, C57BL/6 and Apc^Min/+ /IL-6^-/- mice exhibited near identical muscle weights, indicating that muscle loss does not occur in tumor-bearing animals who had IL-6 ablated. This is consistent with the inhibition of cancer-induced muscle wasting that occurs in response to anti-body mediated neutralization of the soluble IL-6 receptor [42]. When the same three strains of mice were used to test the effects of IL-6 overexpression, muscle wasting occurred in Apc^Min/+ and Apc^Min/+/IL-6^-/- mice but not C57BL/6 mice [38]. The ability of IL-6 to induce wasting when overexpressed in tumor-bearing Apc^Min/+ mice further corroborates its function as a mediator of cachexia development.

Assuming a major regulatory role for IL-6 in the development of cancer cachexia, the issue of which signaling components are important in relaying the signal downstream has been the point of interest. Bonetto *et al.* [36] used microarray analysis to evaluate genome-wide transcript expression in C26 tumor-bearing mice and found evidence of elevated IL-6 along with signal transducer and activator of transcription-3 activity (STAT-3). This was reflected by the upregulated expression of STAT-3 target genes (e.g. acute

phase response genes) in cachectic muscle as well as significantly greater STAT-3 phosphorylation at Y705. The expression of acute phase response genes in skeletal muscle (e.g. fibrinogen) was particularly interesting because the acute phase response to disease or infection arises primarily from IL-6/STAT-3 signaling in the liver [43]. Based on their findings, the authors provided a convincing mechanistic framework for cancer-induced muscle wasting in which the IL-6/STAT-3 pathway also recapitulated in skeletal muscle. As such, increased STAT-3 activity could represent a link between IL-6 and muscle wasting through its ability to upregulate the expression of acute phase response genes in skeletal muscle [36]. This may represent a re-prioritization of protein synthesis in a manner which favors acute phase products that are secreted instead of structural and contractile proteins. Additionally, the same laboratory group reported significantly greater STAT-3 phosphorylation in three other models of cachexia including the B16.F10 melanoma, lewis lung carcinoma, and Apc[Min/+] mice [44]. STAT-3 inhibition by transfection of a dominant negative STAT-3 also significantly reduced muscle loss in C26 tumor-bearing mice [44]. Collectively, it appears that heightened STAT-3 activity plays a critical role in mediating muscle wasting in a broad number of experimental cancer cachexia models.

Insulin-Like Growth Factor-I (IGF-I) Signaling

It has been well established that IGF-I signaling promotes muscle growth and prevents atrophy [45, 46]. Ligation of IGF-I with its receptor initiates a signaling cascade involving the activation of protein kinase B (Akt), mammalian target of rapamycin (mTOR), and 70 kDa ribosomal protein S6 kinase (p70S6k) [45, 47]. Further downstream, the inhibitory protein eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) suppresses the activity of eukaryotic translation initiation factor 4E (EIF-4E) [48]. Phosphorylation of 4E-BP1 by mTOR on the other hand, removes 4E-BP1 inhibition of EIF-4E and allows translational signaling events to proceed [49, 50]. During experimental cancer cachexia, downregulation of the IGF-I signaling pathway has been reported in several studies. For instance, Costelli *et al.* [51] observed decreased circulating and muscle transcript levels of IGF-I concurrently with muscle wasting in rodents bearing the Yoshida AH-130 hepatoma. Similarly, Constantinou *et al.* [37] found decreased IGF-I mRNA along with increased expression of 4E-BP1 in mice undergoing cachexia. The increased levels of 4E-BP1 may inhibit EIF-4E activity, thereby attenuating downstream events leading to protein synthesis. These outcomes appear consistent with reports on muscle IGF-I signaling in Apc[Min/+] mice [42]. Cachectic Apc[Min/+] mice demonstrated lower IGF-I mRNA expression (-28%) and decreased phosphorylation of mTOR (-50%), p70S6k (-37%), and 4E-BP1 (-55%). The suppression of anabolic signaling molecule phosphorylation occurred in concert with a decreased rate of myofibrillar protein synthesis. Taken together, these modifications indicate a cancer-induced alteration in protein turnover (i.e. suppression of protein synthesis) that may contribute to muscle wasting.

Forkhead Box O (FoxO) Activity

The IGF-I pathway inhibits muscle atrophy at least partly through Akt phosphorylation of the FoxO transcription factors [46], three of which are present in skeletal muscle including FoxO1, FoxO3a, and FoxO4 [52]. Phosphorylation by Akt sequesters FoxO and prevents it from translocating to the nucleus where it can drive the expression of atrophy-related genes such as atrogin-1 and muscle ring finger-1 (MuRF1) [53]. These muscle-specific E3 ubiquitin ligases are frequently used markers of ubiquitin-proteasome activity, one of the major proteolytic systems responsible for degradation of cellular constituents [54]. Myofibrillar proteins in particular have been identified as substrates for E3 ligases [55], supporting the role of the proteasome in mediating cancer-induced muscle wasting. Indeed, elevated levels of atrogin-1 or MuRF1

mRNA or protein have been documented in multiple experimental models of cancer cachexia including mice bearing the C26 [6, 56, 57] and lewis lung carcinoma [52] as well as Apc[Min/+] mice [39]. Likewise, increased expression of other components of the ubiquitin-proteasome system has also been reported in experimental cancer cachexia [58, 59].

The regulation of atrogin-1 and MuRF1 gene transcription are believed to be under the control of FoxO in a variety of muscle wasting conditions but also specifically in cancer cachexia [52]. Supporting data can be derived from *in vitro* studies, where exposure of myotubes to C26 conditioned medium upregulated atrogin-1 transcriptional activity (determined from reporter assay) along with an increase in the active, dephosphorylated forms of FoxO1 and FoxO3 [32]. In support of this data, Reed *et al.* [52] found that in mice bearing the lewis lung carcinoma, FoxO transcriptional activity increased ~3 fold, which corresponded with elevated mRNA expression of both E3 ligases. Moreover, injection of a dominant negative FoxO ablated atrogin-1 and MuRF1 mRNA expression while also preventing myofiber atrophy [52]. Taken together, these findings suggest that the activity of FoxO and its target genes represent an important pathway contributing to cancer-induced skeletal muscle wasting.

Proteolysis-Inducing Factor

The tumor-secreted proteolysis-inducing factor (PIF) was isolated and identified by the Tisdale group using the MAC16 murine model of cancer cachexia [31]. Because PIF was also found in the urine of weight-losing cancer patients but not their weight-stable counterparts, the investigators proposed that this tumor-derived product likely induces cachexia in both mice and humans [31]. In a subsequent study, the same group reported that re-introduction of PIF into non-tumor bearing mice caused weight-loss and atrophy of lower limb muscles [60]. The ability of PIF to induce generalized wasting supports its purported role as a cachectic factor [60]. Upon binding to its membrane bound receptor, PIF provides the signal for protein degradation through subsequent activation of nuclear factor kappa B (NF-κB) [61], a transcription factor also involved in transducing the upstream signals provided by inflammatory cytokines (e.g. TNF-α) [62]. The regulation of NF-κB occurs through the inhibitory protein IκB [63]. When bound by IκB, NF-κB is localized in the cytoplasm, and transcriptional activity is suppressed; however, phosphorylation of IκB by IκB kinase (Iκκ) removes this inhibition [64]. This allows nuclear translocation of NF-κB to proceed, resulting in the transcription of its target genes [64]. Interestingly, NF-κB appears to mediate protein degradation through the ubiquitin-proteasome system [61]. Thus, both NF-κB and FoxO appear capable of upregulating proteasome activity in skeletal muscle, which highlights the importance of these transcription factors in regulating cancer-induced muscle atrophy.

Myostatin

Myostatin belongs to the transforming growth factor-β (TGF-β) family of ligands as a skeletal muscle specific member that negatively regulates muscle mass [65]. Mutations in the myostatin gene produce a hypermuscular phenotype observed in a number of different species [66-70]. Moreover, overexpression of myostatin has been shown to reduce muscle mass [71] while pharmacological inhibition of myostatin signaling promotes muscle growth [72, 73]. Recently, myostatin signaling has been implicated as a mechanism that may at least partially contribute to wasting during cancer cachexia [32]. Costelli *et al.* [74] reported significantly elevated muscle expression of myostatin mRNA and protein in rodents demonstrating cachexia. Additional evidence for the potential contribution of this signaling pathway to the development of cachexia can be inferred from studies administering a decoy activin type IIB receptor (ActRIIB) [13, 75, 76]. The cell surface version of

this receptor binds TGF-β ligands such as myostatin and exerts inhibitory effects on muscle growth through activity of the transcription factor Smad 2/3 [77, 78]. Administration of a decoy ActRIIB, however, completely reversed or attenuated cancer-induced muscle wasting in mice as evidenced by improvements in whole body lean tissue and individual muscle masses of the lower limb (i.e. gastrocnemius, soleus, and EDL) [13, 75, 76].

Interestingly, a recent report provided evidence that the C26 tumor commonly used in experimental cancer cachexia serves as a source of secreted myostatin. Lokireddy and colleagues [32] utilized proteomic analysis of collected C26 conditioned medium as well as conditioned medium from excised C26 tumors. In addition to detecting the presence of myostatin protein, the investigators also found elevated levels of Activin A, a related TGF-β superfamily member that can also initiate signal transduction across ActRIIB. The presence of both myostatin and activin was verified with follow-up immunoblot analysis of conditioned medium. Immuno-cytochemical techniques applied to C26 cells and the resected C26 tumor also revealed the expression of myostatin. These findings strongly suggest that myostatin can be considered a tumor-specific product capable of inducing cachexia in the host.

Regarding downstream signaling events, previous *in vitro* work implicates the Smad and FoxO transcription factor families [79, 80]. Using human myotubes, Trendelenburg *et al.* [78] reported that myostatin-mediated atrophy occurred as a result of reduced protein synthesis through inhibition of the Akt/mTOR pathway. Because Akt phosphorylates and maintains FoxO in the inactive state [53], downregulation of this pathway would likely promote FoxO activity. Likewise, Lokirredy *et al.* [80] found that myostatin-treated human myotubes underwent significant atrophy, demonstrated a loss of sarcomeric proteins, showed suppressed Akt phosphorylation, and displayed increased ubiquitin ligase expression. These alterations suggest that myostatin promotes muscle wasting by increasing protein degradation and inhibiting protein synthesis. They also reported the increase in ubiquitin ligase expression and loss of sarcomeric proteins to be dependent on the interactions between Smad 3 and FoxO1. More specifically, the authors suggested that myostatin-mediated muscle atrophy resulted from Smad 3 regulation of FoxO1 such that the latter would be present at increased levels. This was evidenced by the increased phosphorylation of Smad 3 along with elevated protein levels of FoxO1 following myostatin treatment, changes which were blocked by exposure to a Smad 3 inhibitor. Combined with the inhibition of Akt activity, the active, dephosphorylated form of FoxO1 could accumulate and subsequently drive the expression of the ubiquitin ligases. Although this has not been replicated specifically in models of cancer cachexia, previous work has demonstrated the major regulatory role of FoxO in cachectic mice bearing the lewis lung carcinoma [52]. When taking into account the evidence that myostatin may be a tumor-secreted product [32], it is possible that cancer-induced muscle wasting may also arise from Smad and FoxO interactions.

Regenerative Capacity

Because skeletal muscle fibers are post-mitotic, the regenerative process falls under the domain of satellite cells [81]. These muscle stem cells are architecturally positioned between the basement membrane and sarcolemma of the myofiber [82]. In response to regenerative stimuli such as injury, disease, or mechanical loading, satellite cells are activated to produce progeny that can be incorporated into existing myofibers or fuse together to form new myofibers [81, 83]. Some of the major signals that direct satellite cell activity include circulating or local factors (i.e. mitogens), the paired box transcription factors (e.g. Pax7), and the myogenic regulatory factors (e.g. MyoD, myogenin) [83, 84]. Pax7 expression occurs predominantly during quiescence or in the proliferative state whereas the myogenic regulatory factors govern terminal

differentiation [84, 85]. Recent evidence suggests an impairment of the aforementioned myogenic events in cell culture studies. Lokireddy *et al.* [32] observed suppressed proliferation (i.e. cell number) with the addition of C26 conditioned medium to C2C12 myoblasts, suggestive of impaired myogenesis while in a tumor-bearing state. They also reported that exposure to C26 conditioned medium inhibited differentiation as indexed by reduced myotube number, myosin heavy chain content, and MyoD and myogenin protein. Likewise, Guttridge *et al.* [62] reported an absence of myosin heavy chain in addition to a reduction in MyoD mRNA and protein as a result of NF-κB activity in C2C12 cells. The p65 subunit of NF-κB in particular appears to be responsible for the reduced MyoD levels [62]. This impairment of myogenic differentiation *in vitro* agrees with related work done in a murine model of cachexia. Penna and colleagues [86] reported greater immunolabeled Pax7 as well as increased and decreased Pax7 and myogenin protein, respectively, in cachectic muscle obtained from C26 tumor-bearing mice. The authors suggested that the increase in Pax7 expression (as occurs during proliferation) possibly represented an accumulation of satellite cells that because of the concurrent reduction in myogenin levels, lacked the ability to differentiate into muscle precursors. Thus, skeletal muscle wasting in cancer cachexia could be associated with a decreased aptitude of satellite cell progeny to differentiate, thereby reflecting an impaired regenerative capacity.

MECHANISMS OF HMB AND IMPLICATIONS FOR CANCER CACHEXIA

To our knowledge, only a handful of studies have undertaken mechanistic examinations of HMB function specifically as it pertains to cancer cachexia (Table **1**). Overall, they provided some indication that HMB can act on important pathways regulating muscle mass in a manner that could ameliorate cancer-induced muscle wasting. In this section of the review, we provide a brief overview of studies specific to HMB and cancer cachexia with particular attention given to the cellular and molecular aspects of HMB action. We also touch upon other documented mechanisms of HMB that could be favorable in the context of cancer cachexia.

HMB and Cancer Cachexia

Using murine myotubes, Smith *et al.* [87] examined the mechanism of HMB action in the presence of PIF, a secreted tumor-product believed to induce muscle wasting. As expected, exposure to PIF increased the rate of protein degradation relative to control values (+170%). This elevation in protein breakdown occurred in conjunction with increased "chymotrypsin-like" enzyme activity, an indicator of ubiquitin-proteasome activity. In contrast, treatment with HMB attenuated protein degradation, "chymotrypsin-like" activity, and protein expression of ubiquitin-proteasome subunits. Additionally, HMB treatment reduced nuclear localization of NF-κB, the transcription factor shown to mediate the degenerative effects initiated by the PIF signal. Taken together, this *in vitro* model of skeletal muscle provided some indication that HMB can antagonize regulatory elements governing PIF activity and presumably, cancer-induced protein degradation.

In addition to attenuating PIF-mediated protein breakdown, HMB also appears to provide protective effects through its stimulatory action on protein synthesis. Eley and Colleagues [88] utilized PIF-exposed murine myotubes to evaluate the degree by which HMB treatment affected protein synthesis in the face of a cachexia-inducing stimulus. While PIF suppressed protein synthesis by 50%, HMB demonstrated the ability to attenuate this decrease to ~90% of control values. Further, HMB treatment increased phosphorylation of mTOR and p70S6k, alterations that are indicative of activated translational signaling even in the presence of PIF. Downstream signaling events also appear to be positively impacted as reflected by increased phosphorylation of 4E-BP1,

Table 1.   **Effects of HMB provision during experimental cancer cachexia**

Model	Methods	Results	Ref.
Murine myotubes+PIF	50 μmol HMB	Attenuated protein degradation ♥Chymotrypsin-like enzyme activity ♥Ub-proteasome protein expression ♥Nuclear localization of NF-κB	[87]
Murine myotubes+PIF	50 μmol HMB	Attenuated suppression of protein synthesis ♠p-mTOR, p-p70S6k ♠p-4E-BP1 and p-EIF-4G ♥4E-BP1 associated with EIF-4E	[88]
Murine myotubes+ TNF-α	50 μmol HMB	Attenuated protein degradation ♥p-EIF-2α and p-EEF2 ♠EIF-4G and EIF-4E · EIF-4G complex	[89]
MAC16 TB Mice	0.25 g HMB /kg BW	Reduced BW loss, Increased soleus mass ♥Rate of protein degradation ♠Rate of protein synthesis ♥Chymotrypsin-like enzyme activity ♥Ub-proteasome protein expression	[90]
AH-130 TB Mice	4% HMB-enriched chow, 24 days	Increased BW, Attenuated loss of gastrocnemius mass Increased p-mTOR, p-p70S6k	[91]
Walker 256 TB Rats	HMB-supplemented chow, 8 weeks	Increased BW Reduced tumor weight Reduced tumor cell proliferative capacity	[92]
Walker 256 TB Rats	0.32 g HMB /kg BW	Increased survival time	[93]

HMB, β-hydroxy-β-methylbutyrate. PIF, proteolysis-inducing factor. Ub, Ubiquitin. TNF-α, tumor necrosis factor-α. TB, tumor-bearing. BW, body weight. p-, phosphorylated. mTOR, mammalian target of rapamycin. p70S6k, 70 kDa ribosomal protein S6 kinase. EIF, eukaryotic initiation factor. EEF, eukaryotic elongation factor.

decreased amounts of 4E-BP1 associated with EIF-4E, and elevated phosphorylated EIF-4G. Comparable findings were obtained in a similar study by the same group using other catabolic mediators such as TNF-α [89]. These HMB-dependent alterations in the translational machinery would presumably favor protein synthesis and muscle growth.

While *in vitro* examinations of HMB countermeasures generally appear positive, an important consideration is whether not this translates to the intact organism. Smith *et al.* [90] performed follow-up work to their previous *in vitro* study using MAC16 tumor-bearing mice as an experimental model of cancer cachexia. They found that HMB provision to tumor-bearing animals reduced weight loss and increased soleus mass relative to their tumor-bearing counterparts that did not receive HMB. These positive morphological changes may possibly be accounted for by HMB-induced alterations in muscle metabolism, as they also reported decreased rates of protein degradation, proteasome enzyme activity, and protein expression of various proteasome subunits. Coupled with the elevated rate of protein synthesis also observed from the muscle of HMB-administered tumor-bearing animals, it is certainly conceivable that HMB administration could be an effective countermeasure to preserve muscle mass through its regulation of synthesis and degradation pathways. These findings more or less corroborated their prior *in vitro* study of HMB efficacy in myotubes treated with PIF (which was derived from the MAC16 tumor).

Additional evidence for the translation of *in vitro* findings to the intact organism can be derived from the work of Aversa *et al.* [91], who administered HMB for ~3 wks to rats bearing the AH-130 hepatoma, another commonly used experimental model of cachexia. Tumor-bearing rats provided with HMB had significantly greater body weight and an attenuated loss of gastrocnemius mass compared to their tumor-bearing counterparts not administered HMB. They also found phosphorylated mTOR and p70S6k to be enhanced by HMB treatment, suggesting that HMB administration promotes

muscle anabolic signaling, which in turn may account to some degree for the observed preservation of body and muscle weight.

Interestingly, HMB also appears to have direct effects on tumor burden that coincides with the retention of body weight. Nunes and Colleagues [92] reported that rats fed HMB-supplemented chow for 8 wks showed increased body weight whereas those not provided with HMB demonstrated decreased body weight at sacrifice. Animals that consumed HMB also exhibited significantly lower tumor weight (-40%) and reduced tumor cell proliferative capacity *ex vivo* compared to tumor-bearing animals not given HMB. Similarly, Smith *et al.* [90] also noted reduced tumor growth rate in MAC16 tumor-bearing animals provided with HMB, a change that was accompanied by attenuated weight and muscle loss. Because the underlying disease relates to the development of cachexia (i.e. tumor-host interactions), it would be reasonable to expect that the observed anti-tumor activity of HMB could also favorably impact cachexia.

Perhaps most impressively, evidence suggests that HMB provision may induce adaptations that confer a survival advantage. For instance, Caperuto *et al.* [93] reported that 4 wks of HMB administration to rodents inoculated with Walker 256 tumor cells had 100% greater survival time compared to those not provided HMB (28 vs. 14 days). One possible explanation for the increased survival time could be related to HMB effects on muscle mass. Zhou *et al.* [13] previously reported that C26 tumor-bearing mice who preserved muscle mass *via* pharmacological manipulation of the ActRIIB pathway also had significantly greater survival. They suggested that the retention of muscle mass likely accounted for the survival benefit because they did not observe any changes in adiposity or cytokines implicated in the development of cachexia (i.e. IL-6, IL-1, TNF-α). It is difficult to discern whether or not the increase in survival time reported by Caperuto *et al.* could be attributed to HMB effects on muscle mass because muscle weights were not reported. However, it would be reasonable to conclude

based on prior work that HMB may be an effective means to retain muscle mass, therefore, in light of the findings reported by Zhou *et al.*, HMB could possibly prolong survival in rodents through its ability to preserve muscle.

Additional HMB Mechanisms of Relevance

In addition to the mechanisms described in the preceding section, HMB has also been shown to regulate other pathways that may be pertinent to the preservation of muscle mass during cachexia. Kornasio *et al.* [28] used an *in vitro* model of skeletal muscle to evaluate HMB effects on the myogenic program. Treatment of muscle cell cultures with HMB induced alterations suggestive of enhanced myogenesis such as increased cell number, MyoD protein, and myosin heavy chain abundance [28]. They also found that HMB treatment induced IGF-I mRNA expression. Because the development and progression of cancer cachexia may be associated with impaired muscle anabolic signaling and regenerative capacity [32, 51, 86], these HMB-dependent effects may favor muscle retention. Also of interest, Hao and Colleagues [94] found that HMB supplementation decreased markers of apoptotic signaling in aged rats. Apoptosis of myonuclei is believed to be at least partly responsible for the loss of muscle mass associated with aging [95]. Because myonuclei govern a certain cytoplasmic region, it has been suggested that loss of a myonucleus should be accompanied by a corresponding reduction in fiber area to maintain the myonuclear domain, thereby resulting in atrophy [96]. Although the contribution of apoptosis to cancer-induced muscle wasting is not well established, there have been reports of DNA fragmentation and increased Bax/Bcl-2 ratio concurrent with muscle wasting in experimental cancer cachexia, which would be suggestive of apoptosis [97, 98]. HMB may therefore be of value as a countermeasure to preserve muscle through its effect on apoptosis. Moreover, HMB treatment has also been shown to reduce the production of TNF-α and IFN-γ by human peripheral mononuclear blood cells [99]. Since these cytokines have been implicated in the development of cancer cachexia [62], HMB administration may be of value by modulating inflammation. In all, the argument could be made that there is a considerable mechanistic basis for HMB countermeasures because it could potentially target more than one cancer cachexia signaling pathway (e.g. myogenesis, Akt/mTOR pathway, apoptosis, inflammation, and proteasome).

Our own investigations of HMB function in catabolic states associated with aging and caloric restriction also appear to provide some support for the assertion that HMB may be utilized as a countermeasure against cancer cachexia. In very old rats (102 wks) fed HMB-supplemented chow for 16 wks, muscle atrogin-1 mRNA expression significantly decreased compared to age-matched controls [100]. This may be of significance because atrogin-1 is an E3 ubiquitin ligase responsible for the degradation of sarcomeric proteins that appears to upregulated during cancer cachexia. Suppressing the activity of the E3's, and presumably the proteasome, could potentially inhibit the degree of atrophy generated from the cancerous state. This is consistent with the attenuation of protein degradation and decreased expression of atrogin-1 and MuRF1 by HMB in dexamethasone-treated myotubes [101]. Moreover, we also observed an induction of muscle MyoD mRNA expression in mice provided with HMB while being subjected to caloric restriction (+295% vs. control) [102]. The upregulated transcript levels of this myogenic regulatory factor could be interpreted as the stimulation of regenerative events by HMB. It may be possible that parallel events induced by HMB could possibly preserve muscle mass in a cancer-related catabolic environment.

SUMMARY AND CONCLUSIONS

Despite being a major unmet medical need of patients, effective therapies for cachexia have yet to be defined. Recently, considerable work has been devoted to identifying key pathways mediating the development and progression of cachexia, an important first step in formulating countermeasure strategies. It is generally accepted that modifications in protein turnover at least partly contribute to the onset of this profound wasting condition. Based on the limited available body of literature, it appears that supplementation with HMB may be a useful component of multi-modal therapeutic strategies through its ability to target multiple cancer cachexia mechanisms. *In vitro* models appear to be consistent and uniform with respect to HMB efficacy, particularly regarding its propensity for suppressing degradative pathways in addition to stimulating protein synthesis. Importantly, *in vitro* findings appear to translate well to studies on the intact organism. Additional *in vivo* studies in a variety of the available experimental models may provide further information pertaining to the efficacy of HMB countermeasures for cancer cachexia.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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