

Chemoproteomic Identification of Spermidine-Binding Proteins and Antitumor-Immunity Activators

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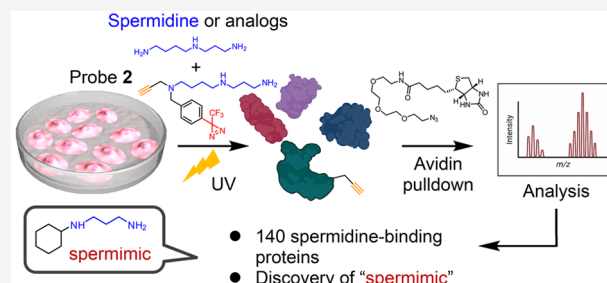
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ABSTRACT: Cancer immune therapies, particularly programmed cell death protein 1 (PD-1) blockade immunotherapy, falter in aged individuals due to compromised T-cell immunity. Spermidine, a biogenic polyamine that declines along with aging, shows promise in restoring antitumor immunity by enhancing mitochondrial fatty acid oxidation (FAO). Herein, we report a spermidine-based chemoproteomic probe (probe 2) that enables profiling of spermidine-binding proteins and screening for small-molecule enhancers of mitochondrial FAO. Chemoproteomic profiling by the probe revealed 140 proteins engaged in cellular interaction with spermidine, with a significant majority being mitochondrial proteins. Hydroxyl coenzyme A (CoA) dehydrogenase subunits α (HADHA) and other lipid metabolism-linked proteins are among the mitochondrial proteins that have attracted considerable interest. Screening spermidine analogs with the probe led to the discovery of compound 13, which interacts with these lipid metabolism-linked proteins and activates HADHA. This simple and biostable synthetic compound we named “spermimic” mirrors spermidine’s ability to enhance mitochondrial bioenergetics and displays similar effectiveness in augmenting PD-1 blockade therapy in mice. This study lays the foundation for developing small-molecule activators of antitumor immunity, offering potential in combination cancer immunotherapy.



INTRODUCTION

Cancer immune therapies, including PD-1 blockade immunotherapy, are ineffective in aged individuals compared to younger counterparts.¹ This discrepancy is closely associated with the age-related decline in energy production, particularly within immune cells, resulting in compromised T-cell immunity in tumor environments. Such impaired antitumor immunity observed in old mice can be ameliorated by stimulating mitochondrial activity² or supplementing spermidine,³ a biogenic polyamine that decreases with age.⁴ Our previous investigation uncovered the role of spermidine in augmenting fatty acid oxidation (FAO) by directly binding and activating hydroxyl coenzyme A (CoA) dehydrogenase subunits α (HADHA), a subunit of mitochondrial trifunctional protein (mTFP) responsible for oxidizing fatty acids to generate NADH.³ The activation of FAO improves the mitochondrial activities and cytotoxic capabilities of T cells, thereby boosting the efficacy of PD-1 blockade immunotherapy in aged mice. Nevertheless, the utility of spermidine *per se* as a therapeutic agent is impeded by its multifaceted actions extending beyond FAO activation, as well as its limited biostability.

Spermidine exerts a broad spectrum of pharmacological effects. Its dietary intake improves age-related impairments within cardiac, nervous, hepatic, and immune systems.⁵

Moreover, spermidine induces fasting-like metabolic changes in mice⁶ and autophagy in multiple cells and tissues.⁷ Although spermidine has been documented to interact with nucleic acids, protein acetyltransferases,⁸ and eukaryotic elongation factor 5A (eIF5A)⁹ in addition to HADHA,³ the precise mechanistic underpinnings governing the diverse actions of spermidine remain incompletely elucidated. Proteomic analysis of spermidine-binding proteins would offer insights into the intricate and multifaceted actions of spermidine. Moreover, the development of drug-like, stable synthetic spermidine analogs could offer an innovative immunotherapeutic application. In pursuit of these goals, we devised chemoproteomic probes for profiling spermidine-binding proteins. The utilization of these probes led to the identification of spermidine binding partners and the discovery of “spermimic,” a biostable analogue of spermidine.

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RESULTS

Design of Spermidine Probes. Spermidine consists of two carbon chains, $(\text{CH}_2)_4$ and $(\text{CH}_2)_3$, sandwiched by nitrogen atoms, forming a characteristic triamine structure (Figure 1A). Our previous investigation underscored the

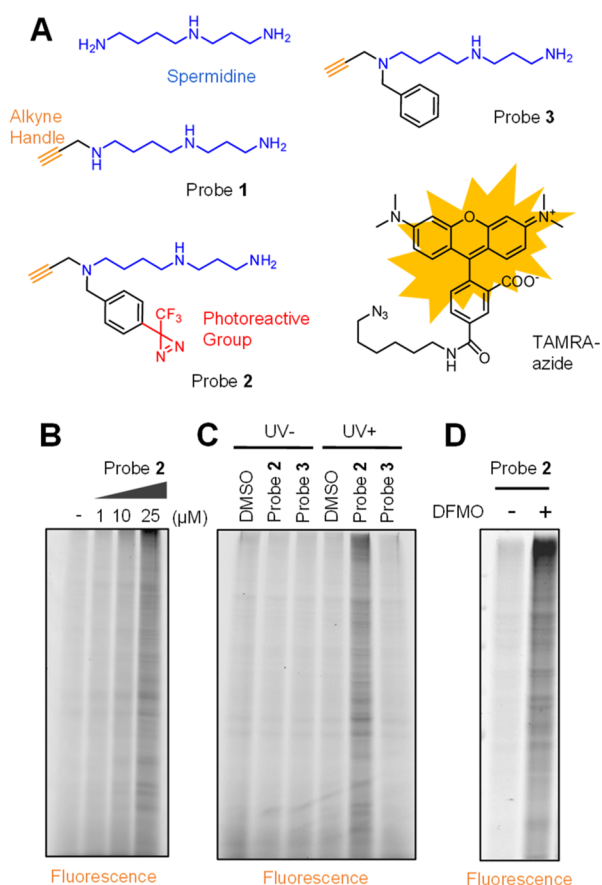


Figure 1. Evaluation of spermidine probes. (A) Chemical structures of spermidine and its probes. (B) HEK293 cells were exposed to varying concentrations of probe 2 for 20 min, followed by a 20 min UV irradiation. Cell lysates underwent click chemistry with TAMRA azide and were subsequently separated by SDS-PAGE for fluorescence scanning analysis. (C) HEK293 cells treated with probe 2 (25 μM) or probe 3 (25 μM) were assessed with and without UV irradiation. (D) HEK293 cells, cultured in the presence or absence of 2.5 mM difluoromethylornithine (DFMO) for 48 h, were later treated with probe 2 (25 μM) followed by UV irradiation.

importance of the $(\text{CH}_2)_3$ moiety in its HADHA activation and consequent impact on FAO.³ In light of this, we designed spermidine probes by functionalizing the $(\text{CH}_2)_4$ segment of the molecule (Figure 1A). Biogenic monoamines have been reported to form covalent bonds with specific proteins through cellular transglutaminases.¹⁰ To test whether spermidine covalently binds to cellular proteins, we initially synthesized probe 1, a simple spermidine derivative endowed with an alkyne handle. Following treatment of HEK293 cells with varied concentrations of probe 1, the resulting cell lysates underwent a click reaction with fluorescent TAMRA-azide. Fluorescent scanning of the SDS-PAGE gel showed no concentration-dependent fluorescent labeling of proteins (Figure S1), indicating a lack of detectable covalent reactions between the probe and cellular proteins.

To analyze the proteins engaging in noncovalent interactions with spermidine in cells, we next designed probe 2, in which spermidine was coupled with an alkyne handle and an aryl-trifluoromethyl diazirine tag (Figure 1A). This photoreactive diazirine tag is often used for the optimal covalent reaction with the interacting partners within a complex biological system.^{11,12} Upon photoirradiation, the tag generates highly reactive carbene species which can readily insert between C–H, C–C, and C–N bonds in its vicinity. HEK293 cells were treated with varied concentrations of probe 2 and UV-irradiated for 20 min. The resulting cell lysates were then subjected to a click reaction with fluorescent TAMRA-azide. Fluorescent scanning of the SDS-PAGE gel showed concentration-dependent labeling of proteins (Figure 1B). In contrast, probe 3, lacking the diazirine moiety, failed to display the UV-induced labeling of proteins, validating the role of the diazirine moiety in the photoreaction (Figure 1C).

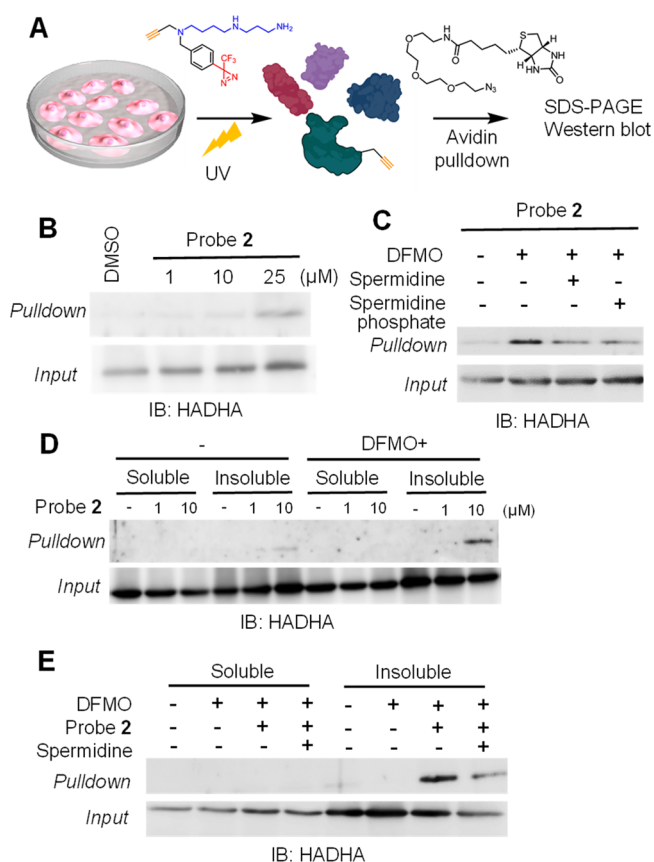
The presence of endogenous intracellular spermidine, estimated at approximately 2 mM, likely competes with probe 2, diminishing the labeling efficacy.¹³ To overcome this hurdle, we employed difluoromethylornithine (DFMO), a well-established inhibitor of polyamine biosynthesis.¹⁴ Addition of DFMO consistently augmented the labeling efficacy (Figure 1D). Under this optimal condition, we evaluated the impact of introducing a phenyl ring to the probe on the proteome labeling (Figure S2). Probe 3 and spermidine competed with probe 2, indicating that the addition of a phenyl ring to spermidine had a minimal effect on protein binding.

To validate the presence of HADHA among the spermidine-binding proteins, we employed biotin-azide instead of TAMRA-azide for the click reaction, followed by the isolation of biotin-labeled proteins using avidin beads and subsequent SDS-PAGE separation (Figure 2A). Western blot analysis with an HADHA antibody revealed dose-dependent labeling of HADHA (Figure 2B). Consistent with expectations, the UV-dependent HADHA labeling was notably potentiated by a 48 h preincubation with DFMO before the photoreaction, and the enhancement was abolished by adding excess amounts of spermidine and spermidine phosphate (Figures 2C and S3).

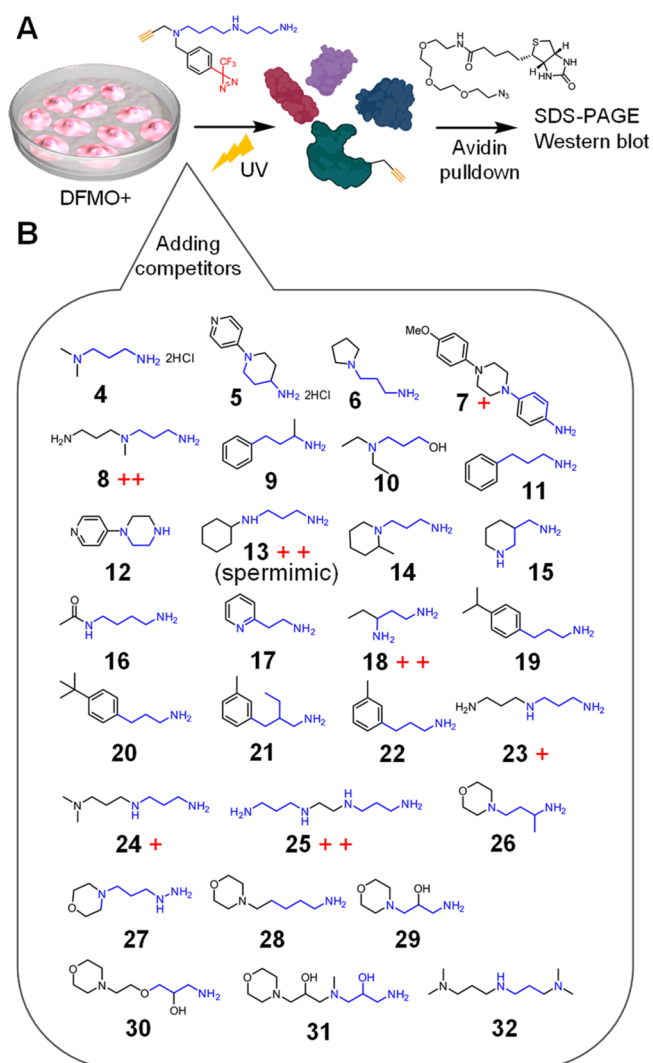
Given that HADHA is a membrane-bound protein,¹⁵ cell lysates after the photoreaction were divided into soluble and insoluble fractions, and these fractions were treated with biotin-azide. In the presence of DFMO, a substantial portion of biotin-labeled HADHA was observed in the insoluble membrane fraction (Figure 2D), and its labeling level was decreased by adding excess amounts of spermidine (Figure 2E). These results suggest that probe 2 predominantly labeled HADHA localized in the mitochondrial membrane.

To further validate probe 2 as a functional spermidine probe, we carried out an *in vitro* enzymatic assay using purified mTFP reconstituted with recombinant HADHA and HADHB. Probe 2 promoted the enzymatic activity of HADHA in generating NADH through the oxidation of 2-*trans*-dodecenoyl-CoA, displaying comparable activation to that of spermidine (Figure S4). Probe 2 was also able to photolabel mTFP, and the labeling was competed with spermidine (Figure S5). These results collectively demonstrate the potential utility of probe 2 in chemoproteomic analysis of spermidine-binding proteins and in the search for synthetic HADHA-binding spermidine analogs.

Discovery of Spermic. Encouraged by the excellent properties of probe 2, we set out to screen 29 distinct



spermidine analogs through the workflow outlined in (Figure 3A). HEK293 cells were preincubated with 2.5 mM DFMO for 48 h, followed by a photoreaction with probe 2 (10 μ M) for 20 min. Compounds 4–32 (1 mM) were individually added 60 min prior to the photoreaction with probe 2. The resulting cell lysates underwent a click reaction with biotin-azide, followed by avidin-pull-down and subsequent HADHA Western blot analysis to assess the competition (Figures S6–S9). The overall results, summarized in Figure 3B, elucidate the requirement of primary and secondary amines, separated by a three-carbon linker, for the binding to HADHA. The four potent compounds (8, 13, 18, and 25) were further evaluated at lower concentrations. While 13 displayed competition even



at 10 μ M which is <1/10-fold lower than spermidine did (Figures 4A,B), 8, 18, and 25 exhibited limited competition at 100 μ M (Figure S10). Additionally, compound 13, in which a cyclohexane ring is attached to the diaminopropyl group, outperformed spermidine in enhancing the enzymatic activity of HADHA (Figure 4C). We thus decided to focus on compound 13 for subsequent studies and designated this simple synthetic molecule as “spermimic”. It is interesting to note that we observed enhanced band intensity induced by compound 27. Such reactivity enhancement of a chemoproteomic probe has been documented in recent studies for both enzyme inhibitors and activators.^{16–18}

Chemoproteomic Profiling. Probe 2 can also be used for chemoproteomic profiling of spermidine-binding proteins

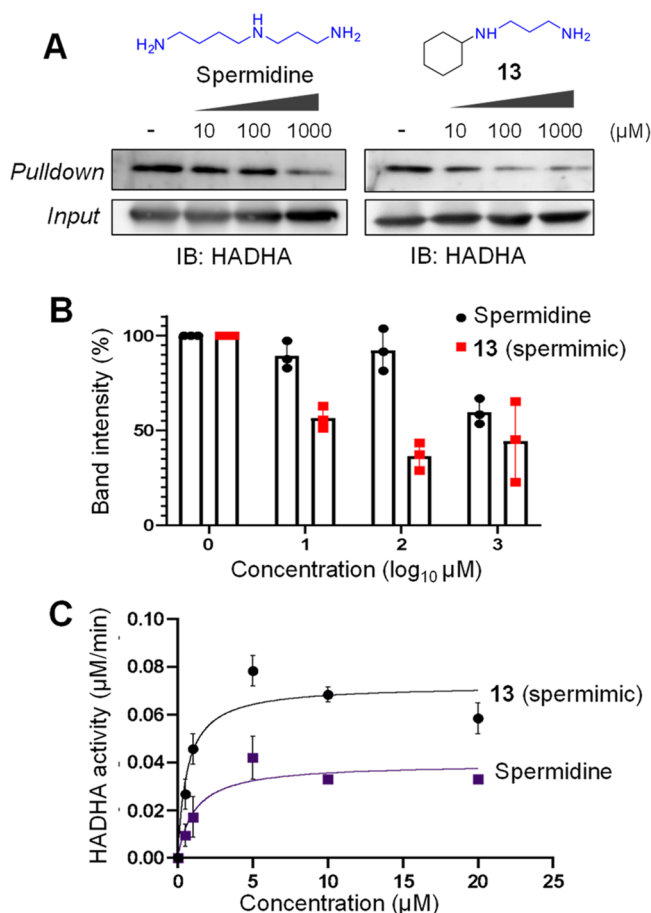


Figure 4. Discovery of compound 13 (spermimic) as an HADHA activator. (A) HEK293 cells were preincubated with 2.5 mM DFMO for 48 h, followed by a photoreaction with probe 2 (10 μM) for 20 min. Spermidine or compound 13 (10–1000 μM) were individually added 60 min prior to the photoreaction with probe 2. Cell lysates underwent click chemistry with biotin-azide, followed by avidin-pulldown and subsequent HADHA Western blot analysis to assess competition. (B) Densitometric quantification of band intensities. Data are represented as mean ± SD ($n = 3$). (C) Impact of compound 13 (spermimic) on *in vitro* HADHA activity. The activity of HADHA (HADHA-HADHB complex) was tracked while exposed to varied concentrations of 13 by measuring UV absorbance at 340 nm, representing the production of NADH. Negligible enzyme activity was observed in the absence of spermidine or 13 (0 μM data). Data are presented as mean ± SEM ($n = 3$).

through the workflow outlined in Figure 5A. Proteins photolabeled with probe 2 were biotinylated and subsequently isolated. The LC-MS/MS analysis of the isolated proteins identified a set of 232 proteins to be labeled by probe 2 (statistically significant at p -value <0.005 and exhibiting >5-fold change compared to the DMSO control) (Figure S11). Out of 232 proteins, the labeling of 140 proteins was significantly competed upon preincubation with 1 mM spermidine before the photoreaction with probe 2 (statistically significant at p -value <0.05 and exhibiting >2-fold change by adding 1 mM spermidine) (Figure 5B and Supporting Excel files 1 and 2). As anticipated, this subset of 140 proteins included HADHA. Interestingly, other reported spermidine-binding proteins (protein acetyltransferases, eIF5A, and HADHB) were not listed among these 140 proteins. It is important to acknowledge that proteins identified through *in*

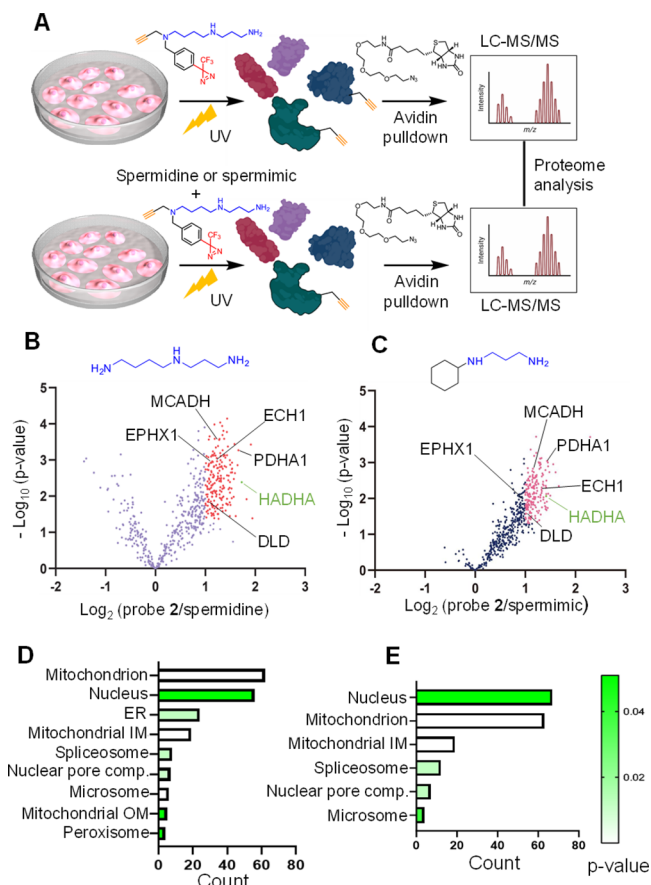


Figure 5. Chemoproteomic profiling of spermidine- and spermimic-binding proteins. (A) Workflow of the chemoproteomic profiling. HEK293 cells were preincubated with 2.5 mM DFMO for 48 h, followed by a photoreaction with probe 2 (10 μM) for 20 min. For identifying selective binding proteins, 1 mM spermidine or 1 mM spermimic was added prior to the photoreaction to displace probe 2 competitively. Cell lysates underwent click chemistry with biotin-azide, followed by avidin-pulldown and subsequent LC-MS/MS analysis. The experiment was conducted in triplicate. (B) Volcano plot [Log₂ ratio (probe 2/spermidine) vs -Log₁₀ p-value]. A p -value cutoff of 0.05 was applied, and a fold change cutoff of 2 [probe/spermidine] was chosen. The proteins that met these criteria are highlighted as red dots. (C) Volcano plot [Log₂ ratio (probe 2/spermimic) vs -Log₁₀ p-value]. A p -value cutoff of 0.05 was applied, and a fold change cutoff of 2 (probe/spermimic) was chosen. The proteins that met these criteria are highlighted as pink dots. (D) Gene Ontology (GO) enrichment analysis (cellular component) was performed for the 140 proteins identified to interact with spermidine. (E) Gene Ontology (GO) enrichment analysis (cellular component) was performed for the 145 proteins identified to interact with spermimic.

vitro biochemical purification and cell-based chemoproteomic capture can vary depending on the experimental conditions of *in vitro* purification.¹⁹ Gene ontology (GO) enrichment analysis (cellular component) highlighted that a majority of the 140 proteins are mitochondrial metabolic proteins (Figures 5D and S12A), consistent with the generally accepted premise that amines tend to accumulate within mitochondria and the notion that spermidine modulates mitochondrial metabolism.

To examine the selectivity of compound 13 (spermimic), we carried out a similar chemoproteome analysis with spermimic. The results revealed a significant overlap among the proteins interacting with both spermidine and spermimic (Figure 5C

and Supporting Excel files 1 and 3). We identified 140 proteins interacting with spermidine and 145 proteins with spermidic, among which 121 proteins were found to overlap. GO enrichment analysis of the 145 spermidic-binding proteins also confirmed a preference to mitochondrial metabolic proteins (Figures S5E and S12B). The overall results indicate that spermidic faithfully mimics spermidine in its protein interactions.

From these mitochondrial metabolic proteins, we selected five representative proteins involved in lipid metabolism, namely, epoxide hydrolase 1 (EPHX1),²⁰ dihydrolopoamide dehydrogenase (DLD),²¹ enoyl-CoA hydratase 1 (ECH1),²² pyruvate dehydrogenase E1 subunit alpha 1 (PDHA1),²³ and medium-chain acyl-CoA dehydrogenase (MCADH).²⁴ Labeling of the five proteins with probe 2 and competition with spermidine or spermidic were confirmed by Western blots (Figures S13 and 6A), validating the results of the chemoproteomic analysis. Remarkably, spermidic demonstrated competition with probe 2 at 10 μ M for these metabolic proteins, similar to the observation for HADHA.

Biostability of Spermidic. Spermidine undergoes rapid enzymatic degradation primarily by serum amine oxidases, yielding cytotoxic acrolein metabolites.²⁵ This inherent low biostability has impeded its potential therapeutic application. In fact, elevated concentrations of spermidine in the medium containing 10% fetal bovine serum (FBS) induced mortality in CD8⁺ T cells, with mitigated cell death observed upon concurrent addition of the amine oxidase inhibitor aminoguanidine (Figure S14). In contrast, identical concentrations of compound 13 (spermidic) exhibited no cytotoxicity toward CD8⁺ T cells. Stability assessment in a medium containing 5% FBS revealed a half-life of 2.8 h for spermidine, contrasting sharply with a significantly prolonged half-life of 501.6 h for spermidic (Figure S15). These results suggest the superior biostability of spermidic.

Antitumor-Immunity Activation by Spermidic. We next set out to assess the impact of spermidic on the bioenergetics of CD8⁺ T cells. Oxygen consumption rates (OCR) were measured at the basal state and after sequential addition of oligomycin (which blocks oxidative phosphorylation-dependent ATP synthesis), carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP which uncouples ATP synthesis from the electron transport chain), and rotenone plus antimycin A (which block electron transport complexes I and III, respectively) (Figure 6B).²⁶ The OCR assessment was conducted in the presence of the amine oxidase inhibitor aminoguanidine to eliminate the effects of the enzymatic degradation of spermidine. Similar to spermidine, spermidic enhanced the spare respiratory capacity (SRC), a measure indicative of mitochondrial metabolic potential represented by OCR uncoupled from ATP production. These results demonstrate that spermidic retained the ability of spermidine to enhance mitochondrial bioenergetics.

Subsequently, we investigated whether spermidic could enhance the PD-1 blockade therapy in mice. Our previous study revealed that oral supplementation of spermidine augments the antitumor activity of PD-L1 monoclonal antibody (PD-L1 mAb) both in young and aged mice.³ Young mice bearing MC38 carcinoma cells were treated with a limited dose of PD-L1 mAb (20 μ g/mouse) along with spermidic or spermidine (Figure 6C). Although the 20 μ g/mouse dose of PD-L1 mAb alone was ineffective in inhibiting tumor growth, the supplementation with spermidic enhanced

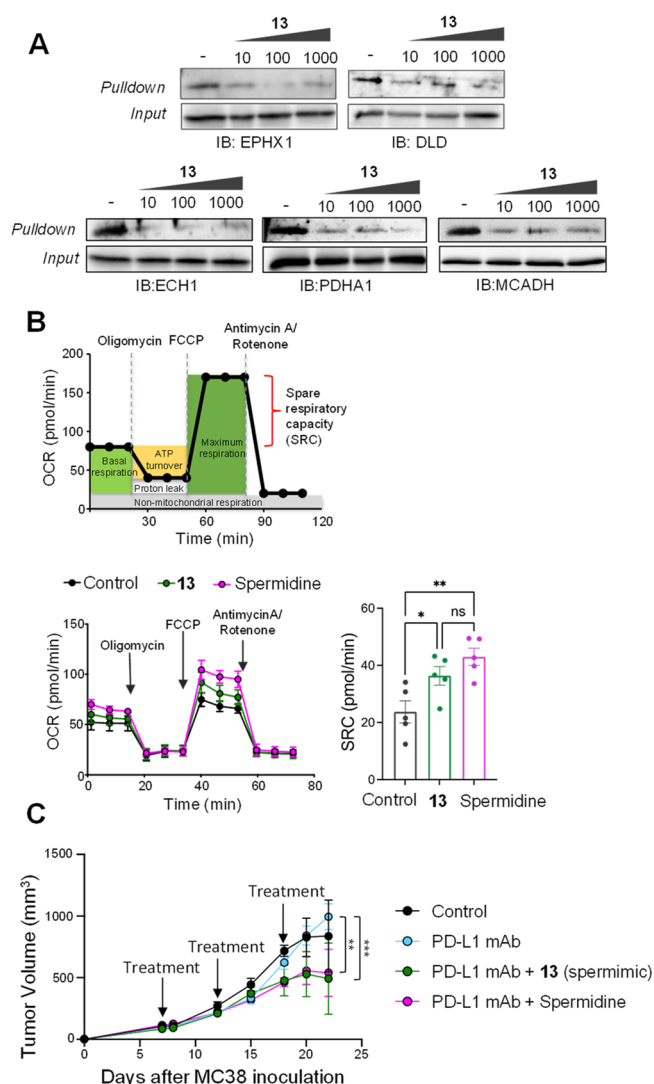


Figure 6. *In vitro* and *in vivo* evaluation of compound 13 (spermidic). (A) Ability of 13 to bind to five representative spermidine-binding metabolic proteins. HEK293 cells were preincubated with 2.5 mM DFMO for 48 h, followed by a photoreaction with probe 2 (10 μ M) for 20 min. Compound 13 (10–1000 μ M) was added 60 min prior to the photoreaction with probe 2. Cell lysates underwent click chemistry with biotin-azide, followed by avidin-pulldown and subsequent Western blot. (B) Effects of 13 (spermidic) on mitochondrial respiration. A representative oxygen consumption rate (OCR) plot is shown. Mouse naive CD8⁺ T cells were stimulated with CD3/CD28 beads in the presence of spermidine or compound 13 for 1 h. Real-time OCR of the cells was measured using Seahorse assay. OCR data are represented as mean $\pm$ SD (n = 5 technical replicates). SRC data are represented as mean $\pm$ SEM (n = 5 technical replicates). (C) *In vivo* evaluation of 13 (spermidic). MC38-bearing mice were treated intraperitoneally with PD-L1 monoclonal antibody (20 μ g/mouse) along with oral administration of compound 13 or spermidine (at 4 mg/kg) on days 7, 12, and 18. Data are represented as mean $\pm$ SEM (n = 5 mice per each group). ** p < 0.01, *** p < 0.005, Two-way ANOVA followed by Tukey's test on day 22.

the antitumor activity at a similar level to spermidine, signifying a comparable therapeutic impact. Nine-week experiments demonstrated that mice treated with spermidic exhibited a higher survival rate compared to control mice, and marginally higher than those treated with spermidine

(Figure S16). It is interesting to note that spermidine did not distinctly outperform spermidine *in vivo*. It is plausible that the high dosage of spermidine used in this study saturated its effect, potentially posing a challenge for spermidine to surpass spermidine's efficacy.

DISCUSSION

In the present study, spermidine probe 2 enabled the identification of 140 proteins engaged in cellular interaction with spermidine and the discovery of spermidine-mimicking compound 13 designated as spermidine. Spermidine augmented the ability of PD-L1 mAb to suppress tumor growth in mice by enhancing mitochondrial bioenergetics. Our study has generated several important questions that will be the basis for further in-depth investigations. Foremost, while spermidine and spermidine activate HADHA,³ what are their impacts on other spermidine-binding proteins, particularly those associated with energy metabolism? We previously showed that the spermidine-induced enhancement of OCR and tumor immunotherapy were reduced in mice with T cell-specific deletion of HADHA, underscoring the importance of HADHA-mediated FAO activation in spermidine's enhanced antitumor immunity. Nevertheless, it is plausible to speculate the involvement of other spermidine-binding proteins, particularly the bioenergy-linked mitochondrial proteins that spermidine binds to, thus contributing to spermidine's enhanced antitumor immunity. Our future genetic studies will also focus on elucidating the precise roles played by these proteins.

Another unanswered question is how spermidine binds to HADHA and other lipid metabolism-related enzymes. The intriguing requirement of the primary and secondary amines, separated by a three-carbon linker, for the binding to HADHA implies the presence of a specific binding cleft tailored to accommodate such a structural motif. Future endeavors focusing on structural elucidation will be pivotal in deepening our comprehension of these interactions and facilitating the subsequent optimization of spermidine for enhanced efficacy and selectivity.

Despite these unanswered questions, our chemoproteomic profiling of spermidine-binding proteins has provided new insights into the intricate actions of spermidine. The discovery of spermidine serves as a starting point for the development of spermidine-based therapeutics for augmenting the PD-1 blockade cancer immunotherapy.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.3c14615>.

Figures S1–S16 and experimental details (PDF)
Supporting Excel file 1, proteomic data (XLSX)
Supporting Excel file 2, proteomic data (XLSX)
Supporting Excel file 3, proteomic data (XLSX)

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Notes

The authors declare no competing financial interest.

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