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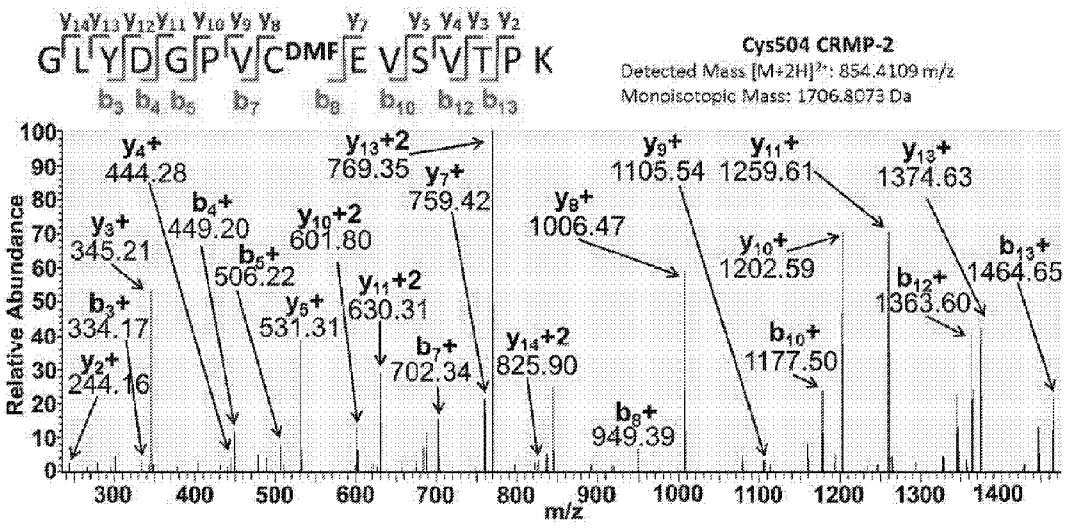
FIG. 2



(57) Abstract: Examination of a test sample to determine the presence or quantity of succination of proteins is described. Examination can be via protein hydrolysis in total succination determination or via enzymatic digestion of isolated proteins and determination of the presence or quantity of modified peptides. The methods can be utilized for determination of excessive succination of lymph system proteins, which can be utilized in prevention or early detection of lymphopenia. Methods can be utilized for test samples of subjects under treatment with dimethyl fumarate suffering from multiple sclerosis. Methods can be utilized as a determination that treatment of the subject with DMF should be slowed or stopped.

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[Continued on next page]

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DETECTION OF FUMARATE ESTER DERIVED MODIFICATION IN A TEST SAMPLE

Cross Reference to Related Application

[0001] This application claims filing benefit of United States Provisional Patent Application Serial No. 62/547,295 having a filing date of August 18, 2017, entitled "Detection of Succination in Lymphocytes," which is incorporated herein by reference for all purposes.

Background

[0002] Multiple sclerosis (MS) is a debilitating disease characterized by the loss of myelin on the nerves of the brain and spinal cord and leads eventually to the death of the axons themselves. It is an autoimmune disease in which the immune cells attack the myelin sheath causing its eventual breakdown around the nerve. As the nerves die lesions that can be monitored by MRI may appear in the brain and spinal cord.

[0003] There is no cure for MS, but several disease-modifying therapies (DMT) exist that can decrease the severity of or delay disease relapses, and most of these act through modulation of the immune system. In 2013 the FDA approved dimethyl fumarate (DMF) (Tecfidera®, Biogen Inc.) for the treatment of relapsing remitting multiple sclerosis (RRMS) and a formulation of DMF (Skilarence®, Almirall) was approved in 2017 in Europe for the treatment of psoriasis. Phase III trials have demonstrated that DMF can significantly reduce the rate of relapse and MRI imaging of patients also suggests that it is neuroprotective, potentially increasing myelin density. While many MS therapies can slow disease progression, the fact that DMF may promote re-myelination is particularly exciting. Alternatives to DMF are also being developed such as, but not limited to, BIIB098 (formerly ALKS 8700), a monomethyl fumarate (MMF) prodrug, that is currently in Phase 3 clinical trials for RRMS.

[0004] DMF is structurally related to the citric acid cycle metabolite fumarate. Fumarates have been shown to react directly with cysteine residues in proteins (known as protein succination) and permanently modify them to affect a protein's structure or function. Specifically, the chemical modification of proteins by fumarate yields S-(2-succino)cysteine (2SC), which has been shown to be increased in adipocytes under diabetic conditions as well as in fumarase-deficient tumors where

fumarate levels are endogenously elevated. Kelch-like ECH-associated protein 1 (KEAP1) has been shown to be a direct target of DMF action (Linker 2011) through similar modification of cysteine residues. This DMF modification of KEAP1 stabilizes a transcription factor, Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), that in turn activates an antioxidant response that increases the cellular capacity to respond to oxidative stress, which is a mediator of the beneficial response in MS patients.

[0005] Despite the documented benefits and the fact that Tecfidera® is reported to be the No. 1 prescribed pill for RRMS in the US, the drug still has a number of mild side effects (gastrointestinal discomfort, flushing) as well as a serious risk of lymphopenia (low lymphocyte counts). This reduction in white blood cells is associated with an increased risk of susceptibility to John Cunningham virus (JC virus) and the development of the deadly brain infection progressive multifocal leukoencephalopathy (PML). Several deaths related to PML in patients being treated with DMF for RRMS have been reported to date. As a result, both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have issued updated safety recommendations to minimize PML risk and the EMA recommends complete blood counts every 3 months to identify patients at risk. Presently, there is no suggested DMF cutoff other than careful monitoring in patients with lymphocyte blood counts lower than 0.5×10^9/L.

[0006] As increasing numbers of patients are treated with DMF for the modulation of RRMS or psoriasis, new reports indicate that current guidelines are insufficient to accurately monitor the risk for PML. There is currently no precise cut-off in white blood cell count that can inform clinicians when a patient should discontinue treatment with DMF or DMF alternatives such as MMF prodrugs. New tests that look at very specific populations of lymphocytes may be one option, but this is not standard clinical practice. Currently there is no accurate way to assess a patient response to such therapy in order to know when therapy should be stopped.

[0007] What are needed in the art are independent measures that assess the level of protein modification (e.g., succination) on a subject due to disease or disease treatment. For instance, a method that can assess the impact of DMF on a patient's immune cells/blood profile through determination of presence or level of protein modification due to the treatment could help the clinician determine when removal from DMF should be considered, even for a short while. Any measurement that could allow more accurate monitoring would be of great benefit to patients

wishing to utilize such a neuroprotective drug (versus other therapies that only slow progression by working on the immunomodulatory aspects of disease therapy).

Summary

[0008] Disclosed are methods for determining the protein modification level in a test sample, and in one embodiment, methods for determining the fumarate ester derived succination level in a test sample. One method can include hydrolyzing proteins of a test sample. The test sample can be one that includes succination protein targets of interest. For instance, the sample can include white blood cells, red blood cells, platelets, total serum, urinary proteins, or a combination of sources of protein targets. A method can also include determining a quantity of modified cysteine (DMF-cysteine, MMF-cysteine, and/or 2SC) in the sample. A method can then include comparing this determined quantity to a control quantity of modified cysteine and, depending upon the comparison, determining that the subject should modify or alter a treatment program, e.g., discontinue treatment with DMF.

[0009] According to another embodiment, rather than hydrolyzing components of a sample, a method can include isolating peripheral blood mononuclear cells (PBMC) of a sample and enzymatically digesting the proteins of the PBMC. Following this, the digested product can be examined to determine the presence or quantity of modified peptides in the sample, the modified peptides including modified cysteine in the peptide sequence. In those embodiments in which the quantity of modified peptides is determined, a method can also include comparing this determined quantity to a control quantity of modified peptides, and depending upon this comparison, determining that a subject should alter a treatment program, e.g., discontinue treatment with DMF.

Brief Description of the Figures

[0010] A full and enabling disclosure of the present subject matter, including the best mode thereof to one of ordinary skill in the art, is set forth more particularly in the remainder of the specification, including reference to the accompanying figures in which:

[0011] FIG. 1 is a table presenting peptide sequences (SEQ ID NO: 1 – 36) derived from proteins modified upon interaction of neural cells with DMF or its metabolite monomethyl fumarate (MMF).

[0012] FIG. 2 illustrates mass spectrometry data of the modified peptide SEQ ID NO: 1 derived from Collapsin Response Mediator Protein 2 (CRMP2).

[0013] Repeat use of reference characters in the present specification and drawings is intended to represent the same or analogous features or elements of the present invention.

Detailed Description

[0014] Reference will now be made in detail to various embodiments of the disclosed subject matter, one or more examples of which are set forth below. Each embodiment is provided by way of explanation of the subject matter, not limitation thereof. In fact, it will be apparent to those skilled in the art that various modifications and variations may be made in the present disclosure without departing from the scope or spirit of the subject matter. For instance, features illustrated or described as part of one embodiment, may be used in another embodiment to yield a still further embodiment.

[0015] In general, disclosed herein are methods for determining the protein modification level of a test sample. More specifically, disclosed methods are directed for examination of a test sample that includes proteins of interest, so as to determine the presence or quantity of fumarate ester derived modification of proteins of the subject. The proteins can include, for example, PBMC proteins (e.g., proteins of mononuclear blood cells, lymphocytes, macrophages, monocytes, etc.) or serum proteins. The methods can be beneficially utilized in one embodiment for determination of excessive fumarate ester derived modification (e.g., succination) of PBMC proteins, which can be utilized in prevention or early detection of lymphopenia. In those embodiments in which a subject is under treatment with DMF, for instance in treatment of MS or other autoimmune disorders (e.g., psoriasis), the method can be utilized as a determination that treatment of the subject with DMF should be slowed or stopped.

[0016] The mode of action of DMF in treatment of MS is understood to be through the KEAP1/Nrf2 pathway. The disclosed method has been developed through realization that KEAP1 is not the only physiological target of DMF, and other targets including the nervous system, lymph system and/or plasma/serum/urinary/tissue proteins may be affected by the drug. For example, a specific cysteine residue on CRMP2 can be modified by DMF (specific mass spectrometry evidence of this is illustrated in FIG. 2). This cysteine is adjacent to several other serine/threonine residues that become phosphorylated as an axon retracts (degenerates) in the progress of MS.

[0017] Without wishing to be bound to any particular theory, it is believed that the modification of this cysteine residue may prevent the phosphorylation of the adjacent residues and thereby may prevent the signal for axon degeneration, acting in a neuroprotective manner. An article (Morinaka et al. Sci Signaling 2011, 26;4(170):ra26) demonstrating that this cysteine needs to be oxidized prior to the phosphorylation signal supports this understanding (as succination by DMF would prevent oxidation).

[0018] In addition to being present in brain cells, CRMP2 is also present in lymphocytes and plays a role in their migration, e.g., in response to viral infections (see, e.g., Varrin-Doyer, et al. J Immunol. 2012 188:1222-33). Thus, the CRMP2 protein is involved in both neural motility and lymphocyte motility – 2 key cell types that are altered in MS disease progression. DMF modification of lymphocyte CRMP2 by succination may alter cell migration properties in response to, e.g., JC virus, and may enable the infection to develop in susceptible individuals.

[0019] Other proteins have also been found capable of being succinated or otherwise modified by fumarate esters. For instance, FIG. 1 describes multiple different brain cell proteins and particular peptides modified by DMF/MMF, at least some of which also present in lymphocytes, blood cells, or other tissues, the modification of which could affect the immune response of an individual. Additional DMF-modified proteins have been described by others (see, e.g., Blewett, et al., Science Signalling, Vol. 9, Iss. 445, Sept. 2016), at least some of which are present in lymphocytes, blood cells, or other tissues, the modification of which could affect the immune response of an individual. For instance, Kornberg et al. (Science. 2018 360:449-453) have demonstrated the specific succination of the protein GAPDH in isolated PBMCs from patients treated with DMF for 3 months, a protein that is also identified in DMF treated neurons in FIG. 1. Thus, in one embodiment, modification of specific proteins can be detected in isolated patient cells.

[0020] Disclosed methods can be beneficially utilized to determine the presence or quantity of succination of blood cell proteins and/or lymphocyte proteins and can be useful in determining potential immune system problems related to protein modification due to interaction with DMF and/or MMF. In particular, methods can be useful for monitoring DMF activity and potential lymphopenia or PML in individuals receiving a drug or prodrug. The disclosed methods can provide a more accurate measure of immune cell or protein susceptibility to excessive fumarate ester

modification as well as individual susceptibility to developing unwanted DMF side effects.

[0021] Protein modification can be measured in isolated lymphocytes or other white blood cells (e.g. peripheral blood mononuclear proteins), plasma proteins other than white blood cells, cerebrospinal fluid, urine or other tissue samples (skin, biopsy material, tumor sample) derived from an individual. A quantified modification level beyond a control value in a subject under treatment with DMF or a prodrug therefore can indicate that the therapy should be slowed or temporarily halted to allow the immune cells time to recover or alternatively to be permanently halted in some embodiments.

[0022] While disclosed methods may be of use with subjects under treatment with DMF or a prodrug therefor, it should be understood that the methods are not limited to this application, and the examination of a sample for the presence or quantity of succinated proteins may be of use in other applications, for instance in cancer patients with suspicious kidney tumors. Such applications could be utilized to determine a 2SC level due to causes other than DMF treatment. For instance, this approach could be useful in diagnosis and/or treatment of disease that demonstrates an endogenous increase in fumarate due to, e.g., tumor growth or metabolism change. By way of example, mitochondrial diseases appear to lead to increased succination in tissues, e.g., brain tissue as discussed by Piroli, et al. (Molecular & Cellular Proteomics, 2016, 15:445-61) for subjects suffering from Leigh Syndrome, and disclosed methods could be useful in diagnosis and/or treatment of such disease states.

[0023] The determination or quantification of succination of proteins in urine, blood, or other sample sources, e.g., lymphocyte protein succination, can be performed in one embodiment by use of isotope dilution mass spectrometry, e.g., using gas chromatograph mass spectrometry (GC-MS) or liquid chromatograph mass spectrometer (LC-MS). For instance, in one embodiment, the absolute quantification of succination (measured by GC-MS) according to methods as described previously (e.g., Alderson 2006, Nagai 2007, Piroli 2016) can be used to accurately measure the overall level of succination in tissue proteins, plasma proteins and urine, among other sample sources.

[0024] In one embodiment, a sample as directly obtained can be examined for presence or quantity of succination. Alternatively, a sample as obtained can be

pretreated, for instance to isolate one or more components of the sample as obtained for examination of succination level. By way of example, a sample can be pretreated to isolate white blood cells of the sample, and this isolated portion of the sample can be examined for presence or quantity of succination.

[0025] In one embodiment, a total serum/plasma sample can be treated in order to precipitate the total protein, for example using an agent that precipitates the protein. The precipitated proteins can then be collected, e.g., by centrifugation, for further analysis of all collected protein of a sample, including PBMC protein. This approach may be beneficial as this can allow for a desired delay from the time of sample collection to succination examination. For instance, serum samples could be frozen with protein extraction and examination for succination carried out at a later time.

[0026] According to one embodiment, total succination quantification of a sample (or one or more components thereof), irrespective of particular proteins affected by the modification, can be carried out via hydrolysis of the proteins and determination of presence/quantity of modified cysteines in the sample. By way of example, whole cell sample (e.g., white blood cells isolated from a sample) or whole protein sample (e.g., total protein separated from a serum or urine sample) can be subjected to lysis. Following, protein from the whole cell or whole protein lysate can be used for analysis of 2SC. For instance, the protein can be precipitated (e.g., with an equal volume of 20% trichloroacetic acid) to form a protein pellet, optionally with removal of other sample components such as lipids. The dried pellet can then be hydrolyzed, for instance by hydrochloric acid (e.g. 6M HCl at 110°C for 24h) and dried (*in vacuo*). The hydrolyzed samples can be further separated (e.g., on C18 columns) to remove particulate matter and elute polar amino acids. N,O trifluoroacetyl methyl ester derivatives can be prepared and analyzed for succination by multiple reaction monitoring (MRM) GC-MS/MS. The parent and daughter ion pairs monitored can include lysine m/z 180 > 69, d8-lysine, m/z 188 > 69; 2SC, m/z 284 >242, U-13C3,15N-2SC m/z 288 > 246, or any other specific ions produced by fragmentation of the above products.

[0027] In one embodiment, liquid chromatography-mass spectrometry methods (LC-MS) may be used to quantify total levels of 2SC. This approach does not require the preparation of the methyl ester derivatives. Instead, free 2SC levels can be detected by methods including but not limited to negative ion mode mass

spectrometry, e.g. similar to methods used by Drusian et al. Cell Reports, 24:1093-1104.e6.

[0028] Quantification of modified cysteine in a sample including proteins can be performed in one embodiment by isotope dilution mass spectrometry based on standard curves constructed from mixtures of known amounts of heavy labeled and natural abundance standard. The amounts of all analytes can be normalized to the lysine content of the samples when detected simultaneously. They can also be normalized to the total protein content or the DNA content of the samples.

[0029] In one embodiment, a quantified sample can be compared to a control sample, so as to determine undesirable cysteine modification levels of the test sample. For example, an acceptable modified cysteine range of a control can be developed from data obtained from a control group comprising individuals that are not under DMF treatment, individuals under DMF treatment with normal blood counts, individuals not suffering from the disease at issue (e.g., mitochondrial disease), or some other suitable standard. A finding that the modified cysteine level of a subjects falls outside of the control range by about 20% or more or, about 10% or more in some embodiments, optionally examined in combination with a total lymphocyte count or a normalized value thereof, can lead to a determination that the individual has an undesirably high level of protein succination, and, in one embodiment, that DMF treatment should be modified.

[0030] For instance, Table 1, below, presents protein succination levels of human blood leukocytes as determined via GC-MS analysis from samples obtained from individuals not under treatment with DMF or any prodrug. These values demonstrate detectable and quantifiable free 2SC in hydrolyzed human white blood cell samples as may be utilized in determining a control level in a quantified testing protocol.

Table 1

Sample No.	2SC (mmol/molLys)
1	0.341
2	1.207
3	0.422
4	0.596

5	0.393
6	0.299
7	0.139
8	0.258
9	0.123
10	0.194
11	0.243
12	0.123
13	0.583
14	0.279
15	0.223
16	0.235
17	1.137

[0031] When conducting a comparison of the modified cysteine level from an individual against those values taken from a control group, the number of individuals utilized in development of the control value may vary, as is generally known in the art. However, in order to be of increased value, a statistically significant number of individuals are generally utilized. Multiple individuals can be utilized in a control group. For example, a control group can include about 10, about 25, about 40 or about 100 individuals or more in order to create a suitable range of control values.

[0032] According to one embodiment, a test sample can be examined for the presence or quantity of particular peptides known to be modified in the presence of DMF and/or MMF. FIG. 1 presents multiple protein targets found in brain cells that DMF binds and modifies. The determination of the presence or quantity of one or more of SEQ ID NOs: 1 – 36 as presented on FIG. 1 can thus be utilized to determine a need to modify DMF treatment.

[0033] According to this embodiment (examples of which are described in more detail in the Examples section, below), a protein sample can be digested into peptides enzymatically and the digested product can be examined to determine the presence or quantity of one or more of SEQ ID NOs: 1 – 36 as peptides of a known size/sequence.

[0034] Based on the known modified proteins of FIG. 1 (or optionally other recognized DMF-modified proteins), a mass spectrometer-based detection regime can be carried out to monitor a sample for the presence or quantity of a particular peptide based upon the mass of the peptide, for instance as provided in the last column of FIG. 1. This approach provides a route to uniquely detect DMF, MMF, and/or 2SC modified proteins in a sample.

[0035] The present disclosure may be better understood with reference to the Examples set forth below.

Example

[0036] Unless otherwise noted, all chemicals were purchased from Sigma/Aldrich Chemical Co (St. Louis, MO). Criterion polyacrylamide gels, nitrocellulose membranes and Precision Plus protein ladder were purchased from BioRad Laboratories (Richmond, CA). Polyvinylidene difluoride (PVDF) membranes and ECL Plus chemiluminescent substrate were from GE Healthcare (Piscataway, NJ). The preparation of the polyclonal anti-2SC antibody has been described previously (Nagai 2007). The following commercial antibodies were used: α-tubulin DM1A, VDAC2 9412 from Cell Signalling Technology, Inc. (Danvers, MA); β-tubulin TUB2.1 from Santa Cruz Biotechnology (Dallas, TX); HNE (HNE-11-S) from Alpha Diagnostics International, Inc. (San Antonio, TX); Ndufs4 2C7CD4AG3 and DJ1 ab4150 from Abcam (Cambridge, MA); VDAC1 clone N152B/23 from Antibodies Inc. (Davis, CA).

Primary Neuron Isolation and Culture

[0037] Primary neurons from newborn rat brain cortices were isolated and cultured using an adaptation of methods described previously. Briefly, postnatal day 1 rats were sacrificed by decapitation, the brains were aseptically dissected and cortices were separated from the rest of the brain in ice-cold Hibernate A medium (Life Technologies) containing 2% (v/v) B-27 supplement (Life Technologies) and 0.5 mM glutamine (Invitrogen). The tissue was minced in fragments of about 1 mm^3 with a scalpel, and subjected to digestion with 2 mg/ml papain (Worthington) in the supplemented Hibernate A medium for 20 min at 30°C in a shaker incubator set at 100 rpm. After thorough trituration through a fire polished Pasteur pipette, the tissue was allowed to settle for 5 min and the supernatant was carefully layered on top of a discontinuous OptiPrep (Sigma-Aldrich) gradient prepared in Hibernate A medium; the layers contained 35, 25, 20 and 15% OptiPrep. The gradient was centrifuged at

800 g for 15 min at room temperature, and layers 1 (15% OptiPrep) and 2 (20%) were discarded. Layer 3 (25%) was collected and added a five-fold volume of Neurobasal A medium (Life Technologies), containing 0.5 mM glutamine and 2% B-27 supplement. After a centrifugation at 500 g for 5 min at room temperature, cells were resuspended in Neurobasal A medium containing 0.5 mM glutamine, 2% B-27 supplement, and 5 ng/ml bFGF (Invitrogen); counted and plated on 24-well plates pretreated with 0.01% poly-L-lysine (Sigma-Aldrich) at a density of 200,000 cells/well. Fifty percent of the media was replaced every third day, with the addition of 5 µM AraC from DIV 3 to inhibit glial proliferation. On DIV 8, cells were left untreated or treated for 24 h with 10 µM or 100 µM dimethyl fumarate (DMF) prepared in Dulbecco's PBS (DPBS) and filtered. On DIV 9, medium was removed, cells were rinsed 3 times with 1 ml DPBS and scraped from the wells after the addition of 250 µl radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, pH 7.4), with the addition of 2 mM diethylenetriaminepentaacetic acid and a protease inhibitor cocktail (P8340, Sigma/Aldrich, St Louis, MO). Homogenization was performed by pulse sonication at 2 watts using a Model 100 sonic dismembrator (Fisher Scientific, Fair Lawn, NJ) for 30 s prior to resting on ice for 30 min in lysis buffer. Protein in the lysates was precipitated with 9 volumes of cold acetone for 10 min on ice. After centrifugation at 3,000 g for 10 min and removal of the acetone, the protein pellet was re-suspended in 150 µl RIPA buffer. The protein content in the different samples was determined by the Lowry assay.

Primary Astrocyte Isolation and Culture

[0038] Primary astrocytes were isolated and cultured from neonatal P1 - P2 Sprague-Dawley rats. Animals were decapitated following which cortices, brainstem, and hippocampi were aseptically dissected in petri dishes filled with ice-cold Hank's Balanced Salt Solution (HBSS; Sigma, St. Louis, MO), HEPES (10 mM; Sigma), and gentamicin sulfate (5 µg/mL; Life Technologies, Carlsbad, CA). The dissected tissue was cut into pieces and dissociated by trypsin treatment (10 µl/mL; Life Technologies, Carlsbad, CA), followed by trituration with sterile glass pipettes. Cells were plated in 24-well plates pre-coated with poly-l-lysine (50 µg/mL; Sigma). Cultures were maintained in DMEM/F12 (Life Technologies) medium supplemented with 10% fetal bovine serum (FBS) and stored in an incubator at 37ºC (5% O_2/95% CO_2). At day *in vitro* 1 (DIV1), cells received a full medium replacement with

subsequent half medium replacements every third day. This protocol results in astrocyte-enriched cultures as confirmed by immunostaining against the astrocytic marker, glial fibrillary acidic protein (GFAP). Cells were harvested at DIV10-12. All animal use was consistent with the guidelines issued by the National Institutes of Health and were approved by the University of South Carolina Institutional Animal Care and Use Committee.

N1E-115 Cell Culture

[0039] N1E-115 cells (subclone N1E-115-1 neuroblastoma cells) were obtained from Sigma (08062511, St. Louis, MO). The cells were grown and expanded in non-differentiation medium (NDM): 90% DMEM (Gibco, Grand Island, NY) with 25 mM glucose, no pyruvate, 25 mM HEPES, 4 mM glutamine and 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA). At 80% confluence, the cells were differentiated into neurons in the presence of 2% FBS and 1.25% dimethyl sulfoxide in DMEM for 5 days. In addition to the assessment of the neuronal phenotype by light microscopy, the detection of synaptophysin protein levels was used to confirm successful differentiation. During the final 6 or 24 h of differentiation the cells were treated with 10-100 μM DMF and harvested as described above for primary neurons, with the addition of phosphatase inhibitors to the DPBS washes and the RIPA buffer used for scraping to prevent dephosphorylation of CRMP-2. For some experiments, cells were treated with recombinant 1-3 nM mouse Semaphorin 3A Fc chimera protein (R&D Systems, Inc., Minneapolis, MN) for 10-30 min, or with 100 μM hydrogen peroxide for 10-15 min before harvesting the cells. Homogenization, acetone-precipitation, resuspension and protein determination in the samples was performed as described for primary neurons.

Lentiviral Transduction of 3T3-L1 fibroblasts

[0040] TRC2 *Fh1* shRNA, clone- TRCN0000246831 or SHC202 MISSION TRC2 pLKO.5-puro non-mammalian shRNA control plasmids (Sigma/Aldrich, St. Louis, MO) were used to generate the lentiviral vectors. The vectors also contained a puromycin resistance gene. 15 μg vector plasmid, 10 μg psPAX2 packaging plasmid (Addgene 12260, Cambridge, MA), 5 μg pMD2.G envelope plasmid (Addgene 12259, Cambridge, MA) and 2.5 μg pRSV-Rev plasmid (Addgene 12253, Cambridge, MA) were transfected into 293T cells. The filtered conditioned medium was collected and stored at -80°C until use.

[0041] 3T3-L1 fibroblasts (ATCC) were incubated overnight with 150 µL of filtered conditioned medium containing *Fh1* shRNA or control lentivirus. Successfully transduced fibroblasts were selected using 1 µg/mL puromycin. The selected fibroblasts were propagated in the presence of puromycin until confluent and harvested in RIPA buffer as described above. Successful knockdown of fumarase expression was determined by immunoblotting and fumarate levels were determined by GC-MS as described previously (Piroli 2016).

Saponification of Fumarate Esters

[0042] 60 µg of protein from control and DMF treated cell lysates was incubated with 80% dimethyl sulfoxide (DMSO), 6 mM potassium hydroxide (KOH), and 1 mM EDTA at room temperature for 30 minutes, with vortexing at 5-minute intervals. The pH was adjusted to 7, and the protein was precipitated with 90% acetone before being re-suspended in 40 µL RIPA buffer. The pH was again adjusted to 7 prior to gel electrophoresis and immunoblotting.

One-dimensional PAGE and Western Blotting

[0043] Western blotting to detect protein succination (2SC), fumarase, CRMP2 (phosphorylated forms and total levels), Haem oxygenase -1(HO-1, Enzo Life Sciences), mitochondrial markers Ndufb8 (complex I, MitoSciences, Eugene, OR), 30 kDa subunit (complex II, MitoSciences), succinate dehydrogenase a (complex II and Krebs cycle, Cell Signaling Technology, Danvers, MA), core 2 subunit (complex III, MitoSciences), alpha subunit (complex V, MitoSciences) and fumarase (Krebs cycle, Cell Signaling Technology), and alpha and beta tubulin (Santa Cruz Biotechnology, Inc., Dallas, TX) was performed as described previously, after separation of the proteins by SDS-PAGE (J Invest Dermatol. 2007 Apr;127(4):835-45). For protein identification purposes gels were stained with Coomassie brilliant blue following electrophoresis to allow band isolation and mass spectrometry (see below). In some cases, membranes were stripped with 62.5 mM Tris, pH 6.8, containing 2% SDS and 0.7% 2-mercapto ethanol for 20 min at 65°C prior to re-probing with a different antibody.

Protein Identification from SDS-PAGE gel bands by LC-MS/MS

[0044] For the identification of succination sites, 60 µg of protein from primary neurons, 200 µg of protein from primary astrocytes or 120 µg of protein from differentiated N1E-115 neurons were resolved by SDS-PAGE, and the gels were stained with Coomassie Brilliant Blue. After destaining, all protein bands were

excised from the gels and subjected to in-gel digestion with trypsin. Briefly, proteins were reduced with 10 mM dithiothreitol and alkylated with 170 mM 4-vinylpyridine. Trypsin digestion was carried out overnight at 37°C in the presence of 1.5 ng sequence grade modified trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate. After gel extraction, the peptides were re-suspended in 1% acetic acid and analyzed on a Dionex Ultimate 3000-LC system (Thermo Scientific, Rockford, IL) coupled to a Velos Pro Orbitrap mass spectrometer (Thermo Scientific, Rockford, IL). The LC solvents were 2 % acetonitrile/0.1 % formic acid (Solvent A) and 80 % acetonitrile/0.1 % formic acid (Solvent B). At 4 minutes the trap column, a 2 cm Acclaim PepMap-100 column (Thermo Scientific) was put in line with the analytical column, a 75 µm C18 stationary-phase LC PicoChip Nanospray column (New Objective, Inc., Woburn, MA). The peptides were eluted with a gradient from 98%A:2%B to 40%A:60%B over 30 minutes, followed by a 5 minute ramp to 10%A:90%B that was held for 10 minutes. The Orbitrap was operated in data-dependent MS/MS analysis mode and excluded all ions below 200 counts. Following a survey scan, 8 precursor ions were selected for further MS/MS. The data-dependent acquisition (DDA) data were analyzed using Proteome Discover 1.4 software with SEQUEST search engine against the uniprot_ref_mouse database (2014-10-03 version, 52,474 proteins) or uniprot_ref_rat database (2011-5-11 version, 39765 proteins) with X_{corr} >1 validation (+2) and an allowance for 2 missed cleavages. For all identifications the spectra were manually inspected to confirm identity and incorrect identifications were discarded. To further confirm the succinated proteins that were identified in DDA mode, multiple reaction monitoring (MRM) was used to monitor select succinated peptide masses of interest for CID (collision-induced dissociation)-MS/MS analysis at a resolution of 7500. The CID-MS/MS data was sequenced manually using Thermo Xcalibur 2.2 software to confirm the modified peptides. No fixed modifications were considered. The variable modifications of methionine oxidation (M^{OX}), proline hydroxylation (P^{ox}) cysteine pyridylethylation (C^{PE}, 105.058) or cysteine succination (C^{2SC}) by fumarate (116.011), cysteine succination by monomethyl fumarate (C^{MMF}, 130.026) or cysteine succination by dimethyl fumarate (C^{DMF}, 144.042) were considered with a mass tolerance of 15 ppm for precursor ions and a mass tolerance of 10 ppm for fragment ions. The mass spectrometry proteomics data have been deposited to the

ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier.

Experimental Design and Statistical Analysis

[0045] Analyses were performed with a minimum of 3 independent biological replicates per group (n=3-8). Data are summarized throughout as mean ± standard error and are plotted using SigmaPlot 11 software (Systat Software, Inc. San Jose, CA) and Prism 4 (GraphPad Software, La Jolla, CA). Statistical analyses were performed using SigmaPlot 11 and Prism 4. When two groups were compared, the Student "t" test was used. Differences between more than 2 groups were analyzed using one-way ANOVA with the Student-Newman-Keuls post-test. In all cases, $P<0.05$ was considered statistically significant.

Results

[0046] In order to detect succinated proteins in neurons treated with DMF for 24 hours, a previously developed procedure was employed (Manuel, 2013) using alkaline hydrolysis to remove the ester and permit immunological detection of succination. In the absence of ester hydrolysis there was limited detection of succinated proteins in rat primary neurons using the anti-2SC antibody, with only one band ~50 kDa showing a significant increase in intensity following 100 μM DMF treatment. This indicated that at least one or both of the methyl groups had not been removed by intracellular esterases and was preventing interaction with the anti-2SC antibody that recognizes the S-(2-succino)cysteine epitope. To ensure that the succinated proteins detected were solely of neuronal origin, N1E-115 neuroblastoma cells were differentiated to a neuronal phenotype (confirmed by increased synaptophysin content) and treated with DMF for 24 hours. The hydrolysis of the ester in the presence of KOH facilitated the detection of a large number of succinated proteins. Fibroblasts that had *fumarase* knocked down using a lentiviral mediated shRNA approach to increase endogenous fumarate levels were used as a positive control for succination. The intensity of succinated proteins in the DMF treated neurons versus the positive control indicated that fumarate esters readily enter cells and react with a wide range of protein thiols. Both DMF and MMF are significantly more reactive than fumarate itself and are capable of modifying intracellular proteins before they are demethylated. Since DMF is known to modify thiols in Keap1 leading to the induction of antioxidant response proteins such as

heme oxygenase 1 (HO-1), it was confirmed that DMF treatment significantly induced HO-1 expression.

[0047] In order to identify the succinated proteins in DMF treated neurons LC-MS/MS was used and results were searched for the succination of protein thiols by either DMF (C^{DMF}), or the demethylated metabolites MMF (C^{MMF}) and fumarate (C^{2SC}). This proteomic approach allows for confirmation of the exact sites of succination and it does not require the base hydrolysis of the ester that can also result in a partial loss of protein. As noted in FIG. 1, all of the proteins identified were succinated by DMF or MMF, rather than 2SC, suggesting that both of these fumarate esters reacted with intracellular proteins more rapidly than they could be converted to fumarate. In primary rat neurons the identity of 4 succinated proteins was confirmed, and in N1E-115 neurons a total of 15 protein subunits modified by either DMF or MMF was confirmed. Since the N1E-115 cultures were devoid of any glial cells that may be present in primary neuronal cultures, the identified proteins reflect true neuronal targets of fumarate ester modification. Since fumarate esters were previously shown to induce Nrf2 in glial cells *in vivo* (Linker 2011), additional protein targets of DMF-mediated succination in primary astrocyte cultures were also investigated. FIG. 1 presents the results confirming the detection of 11 succinated protein subunits in primary rat astrocytes after 24 hours treatment with up to 100 μM DMF. Overall, this targeted approach identified 24 distinct protein subunits in both neurons and astrocytes that were directly modified by either DMF or MMF.

[0048] The chemical modification of several cytoskeletal proteins was observed in all cell types examined (FIG. 1). In astrocytes this included abundant cytoskeletal proteins such as Glial Fibrillary Acid Protein (GFAP) and vimentin, as well as cofilin-1, a dynamic regulator of actin polymerization.

[0049] Strikingly, all three cell preparations confirmed succination of the Collapsin Response Mediator 2 (CRMP2) peptide GLYDGPVCEVSVTPK (SEQ ID NO: 1) by either MMF or DMF (FIG. 1). The y_8 and b_8 fragment ion designations in the annotated spectrum shown in FIG. 2 confirm the designation of Cys504 as the site of succination by DMF in primary rat neurons (+2 charge-state, 854.4109 m/z). The presence of pyridylethylated Cys504, representative of non-succinated cysteine was also detected in the primary rat neurons (+2 charge-state, 834.9182 m/z).

[0050] While certain embodiments of the disclosed subject matter have been described using specific terms, such description is for illustrative purposes only, and

it is to be understood that changes and variations may be made without departing from the spirit or scope of the subject matter.

WHAT IS CLAIMED IS:

1. A method for determining a succination level in a test sample, comprising:

hydrolyzing proteins of a test sample;

determining the presence or quantity of modified cysteines in the hydrolyzed proteins, wherein the modified cysteines have been modified by one or more of dimethyl fumarate, monomethyl fumarate, or fumarate.

2. The method of claim 1, the method comprising determining the quantity of modified cysteines in the hydrolyzed sample, the method further comprising comparing the determined quantity to a control quantity.

3. The method of claim 1 or claim 2, the method further comprising isolating one or more components from the test sample prior to hydrolyzing the proteins of the test sample.

4. The method of claim 3, the one or more components comprising peripheral blood mononuclear cells.

5. The method of claim 4, the peripheral blood mononuclear cells comprising lymphocytes, macrophages, monocytes, or a combination thereof.

6. The method of any one of claims 1 to 5, wherein the modified cysteines have been modified by dimethyl fumarate and/or monomethyl fumarate.

7. The method of any one of claims 1 to 6, wherein the test sample is obtained from a subject that has been diagnosed with an autoimmune disorder and treated with dimethyl fumarate or a monomethyl fumarate prodrug.

8. The method of claim 7, wherein the autoimmune disorder is multiple sclerosis.

9. The method of any one of claims 1 to 8, wherein the step of determining the presence or quantity of the modified cysteines comprises isotope dilution mass spectrometry.

10. The method of any one of claims 1 to 9, the sample comprising blood, cerebrospinal fluid, urine or a tissue biopsy.

11. A method for determining the succination level in a test sample, the method comprising:

isolating peripheral blood mononuclear cells of the sample;

lysing the peripheral blood mononuclear cells;

enzymatically digesting proteins of the lysed peripheral blood mononuclear cells; and

determining the presence or quantity of one or more modified peptides in digested proteins, the one or more modified peptides including at least one cysteine modified with dimethyl fumarate, monomethyl fumarate, or fumarate.

12. The method of claim 11, the one or more modified peptides comprising one or more of SEQ ID Nos: 1 – 36.

13. The method of claim 11, the one or more modified peptides comprising SEQ ID NO: 1.

14. The method of any one of claims 11 to 13, the method comprising determining the quantity of the one or more modified peptides, the method further comprising comparing the quantity to a control quantity.

15. The method of any one of claims 11 to 14, the peripheral blood mononuclear cells comprising lymphocytes, macrophages, monocytes, or combinations thereof.

16. The method of any one of claims 11 to 15, wherein the modified cysteines have been modified by dimethyl fumarate and/or monomethyl fumarate.

17. The method of any one of claims 11 to 16, wherein the test sample is obtained from a subject that has been diagnosed with an autoimmune disorder and treated with dimethyl fumarate.

18. The method of claim 17, wherein the autoimmune disorder is multiple sclerosis.

19. The method of any one of claims 11 to 18, wherein the step of determining the presence or quantity of the modified cysteines comprises mass spectrometry.

20. The method of any one of claims 11 to 19, the sample comprising blood, cerebrospinal fluid, or urine.

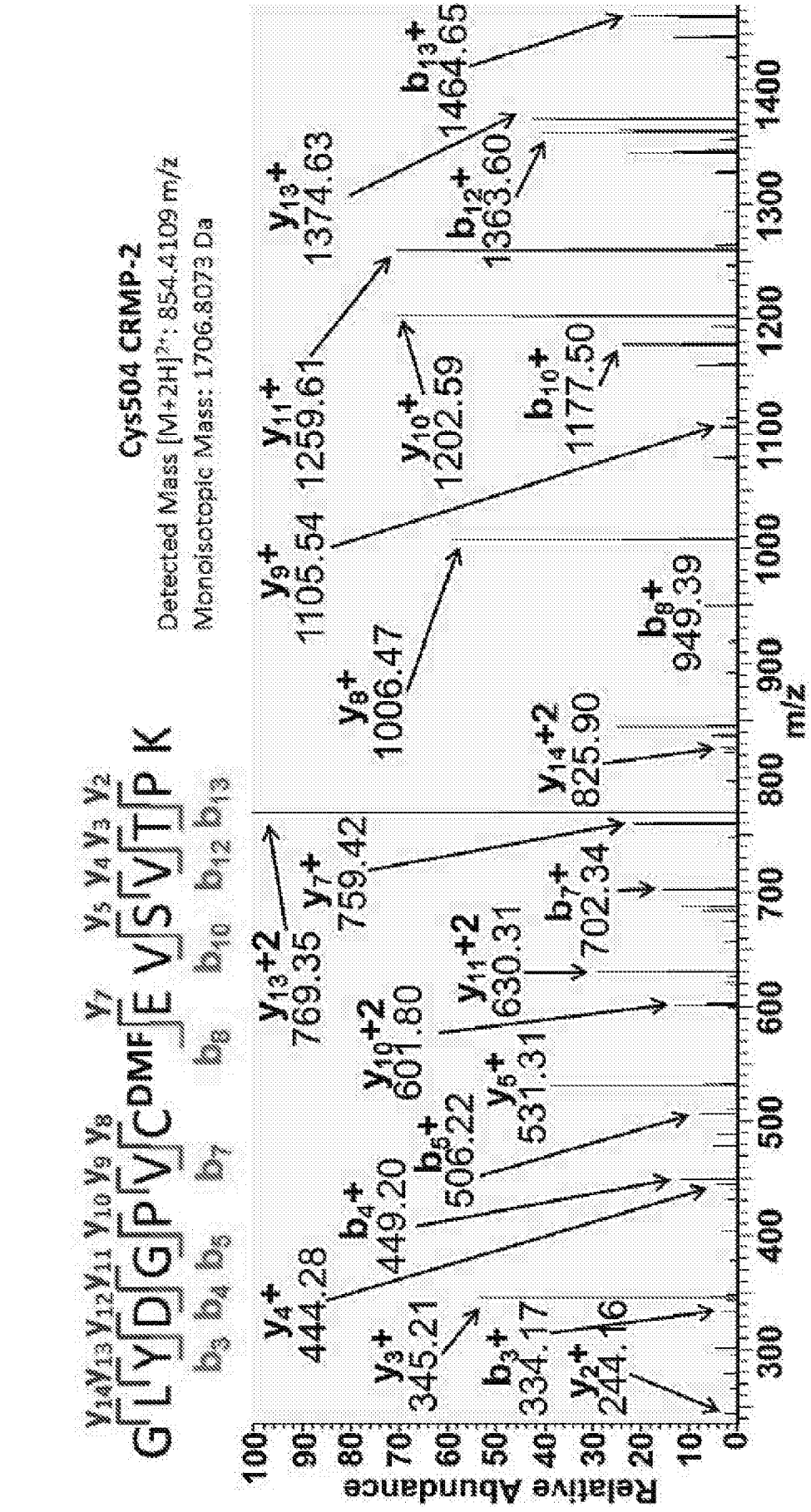
FIG. 1

Protein Name	SEQ ID NO	Modified Sequence	Succination Site	Modified Peptide PSMs	Xcorr	m/z	charge	MH+ [Da]
Primary Neurons								
Collapsin Response Mediator Protein 2	1	GLYDGPVC^{DMF}EVSVTPK	Cys 504	2	1.9	854.4096	2	1707.812059
Vimentin	2	QVQSLTC^{DMF}EVDALK	Cys 228	3	2.42	789.3899	2	1577.772631
Guanine nucleotide-binding protein G(o) subunit alpha	3	M^{OX}VC^{DMF}DVVSR	Cys 108	1	2.56	534.7373	2	1068.467333
Annexin A1	4	QAC^{DMF}YIEK	Cys 12	2	1.21	499.7269	2	998.4465808
Primary Astrocytes								
Filamin alpha	5	YTPC^{MMF}GAGSYTIM^{OX}VLFADQATPTSPIR	Cys 841	1	1.85	969.4722	3	2906.402134
Myosin-9	6	C^{MMF}NGVLEGIR	Cys 694	10	1.21	545.7675	2	1090.524828
Heat shock protein HSP 90-alpha	7	VFIM^{OX}DNC^{DMF}EELIPEYLNFIR	Cys 375	3	2.24	840.0648	3	2518.180088
Heat shock protein HSP 90-beta	8	VFIM^{OX}DSC^{DMF}DELIPEYLNFIR	Cys 366	4	1.49	1239.089	2	2477.171678
Collapsin Response Mediator Protein 2	9	GLYDGPVC^{MMF}EVSVTPK	Cys 504	2	3.08	847.402	2	1693.796801
	10	GLYDGPVC^{DMF}EVSVTPK	Cys 504	3	1.83	854.4108	2	1707.814501
Vimentin	11	QVQSLTC^{DMF}EVDALK	Cys 328	3	2.25	789.3909	2	1577.774706
	12	QVQSLTC^{MMF}EVDALK	Cys 328	3	1.65	782.3807	2	1563.754198
Glial fibrillary acidic protein	13	QLQALTC^{MMF}DLESLR	Cys 292	1	1.37	810.4024	2	1619.797533
Annexin A1	14	GDRC^{DMF}EDM^{OX}SVNQDLADTDAR	Cys 190	1	2.04	757.6424	3	2270.912815
	15	QAC^{DMF}YIEK	Cys 12	1	1.06	499.7257	2	998.4441394
Cofilin-1	16	HELQANC^{MMF}YEEVKDR	Cys 139	1	4.86	621.9409	3	1863.808201
Peptidyl-prolyl cis-trans isomerase A	17	VC^{DMF}FELFADK	Cys 21	1	1.95	608.2839	2	1215.560594
Fatty acid-binding protein,brain	18	TQC^{DMF}TFK	Cys 56	3	2	4361. 958	2	871.3869494
NIE Neurons								
ATP-citrate synthase	19	YIC^{MMF}TTSAIQNR	Cys 20	2	1.57	700.3239	2	1399.640551
Ubiquitin carboxyl-terminalhydrolase isozyme L1	20	NEAIQAAHDSVAQEGQC^{MMF}R	Cys 152	6	5.87	686.3021	3	2056.891819

FIG. 1 (cont.)

	21	NEAIQAAHDSVAQEGQC[DMF]R	Cys 152	4	4.54	690.9748	3	2070.910007
Isoform Mt-VDAC1of Voltage-dependent anion-selective channelprotein 1	22	YQVDPDAC[MMF]FSAK	Cys 245	11	1.67	737.3118	2	1473.610765
T-complex protein 1subunit beta	23	HGINC[MMF]FINR	Cys 289	2	1.03	602.2833	2	1203.559374
T-complex protein 1subunit alpha	24	LAC[MMF]KEAVR	Cys 125	3	1.16	510.2606	2	1019.514086
Elongation factor 2	25	STLTDSLVC[MMF]K	Cys 41	1	1.95	5987. 895	2	1196.571825
Heat shock protein HSP 90-beta	26	VFIM[OX]DSC[MMF]DELIPEYLNFIR	Cys 366	2	1.16	1232.073	2	2463.138719
	27	VFIM[OX]DSC[DMF]DELIPEYLNFIR	Cys 366	3	1.98	826.3831	3	2477.164843
Heat shock protein HSP 90-alpha	28	VFIM[OX]DNC[DMF]EELIPEYLNFIR	Cys 375	5	2.77	840.0639	3	2518.177158
Glyceraldehyde-3-phosphate dehydrogenase	29	AAIC[MMF]SGK	Cys 22	3	1.37	390.1814	2	779.3555163
IgE-binding protein	30	QQC[MMF]AER	Cys 187	1	1.4	432.678	2	864.3488635
	31	LQGPPYAESPPC[MMF]VVR	Cys 181	2	2.09	871.9227	2	1742.838182
Transgelin-2	32	NM[OX]AC[MMF]VQR	Cys 124	2	2.24	484.2015	2	967.3957995
Collapsin Response Mediator Protein 2	33	GLYDGPVC[MMF]EVSVTPK	Cys 504	13	3.45	847.4002	2	1693.79326
Asparagine synthetase [glutamine-hydrolyzing)	34	ETFEDC[DMF]NLLPK	Cys 455	3	2.36	726.838	2	1452.668383
Tubulin alpha-1A chain	35	TIQFVDWC[DMF]PTGFK	Cys 347	2	2.6 4	843.3975	2	1685.787767
Tubulin beta-SA chain	36	TAVC[DMF]DIPPR	Cys 354	1		558.6467	2	1115.5401

FIG. 2


Cys504 CRMP-2
Detected Mass [M+2H]2+: 854.4109 m/z
Monoisotopic Mass: 1706.8073 Da
G L Y D G P V C DMF E V S V T P K
Relative Abundance
m/z
y4+ 444.28
y3+ 345.21
b3+ 334.17
y2+ 244.16
b4+ 449.20
b5+ 506.22
y10+2 601.80
y13+2 769.35
y7+ 759.42
y5+ 531.31
y11+2 630.31
b7+ 702.34
y14+2 825.90
y8+ 1006.47
b8+ 949.39
y9+ 1105.54
y11+ 1259.61
y10+ 1202.59
b10+ 1177.50
y13+ 1374.63
b12+ 1363.60
b13+ 1464.65

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/46861

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C07K 14/72, 14/61; G01N 33/68 (2018.01)

CPC - C07K 14/72, 14/61, 14/505, 14/56, 1/1133; G01N 33/6815, 33/68, 33/5008

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	PIROLI, GG et al. Identification of Protein Succination as a Novel Modification of Tubulin. Biochemical Journal, 01 September 2014, Vol. 462, No. 2, pages 231-245; abstract; page 3, third paragraph; page 5, third paragraph; page 6, second paragraph; page 9, third paragraph; page 10, first paragraph; page 11, third paragraph	1, 2, 3/1-2 ---------- 4/3/1-2, 5/4/3/1-2, 11-13, 14/11-13
Y	US 2013/0190201 A1 (ADVANCED TECHNOLOGIES AND REGENERATIVE MEDICINE, LLC) July 25, 2013; abstract; paragraphs [0006], [0016], [0017], [0123], [0192], [0198], [0241]	4/3/1-2, 5/4/3/1-2, 11-13, 14/11-13
Y	US 2010/0047817 A1 (OTTENS, AK et al.) February 25, 2010; paragraphs [0049], [0126]; SEQ ID NO: 7	12, 13, 14/12-13
A	BLATNICK, M et al. Succination of Proteins by Fumarate: Mechanism of Inactivation of Glyceraldehyde-3-Phosphate Dehydrogenase in Diabetes. Annals New York Academy of Science, April 2008; Vol. 1126; pages 272-275; entire document	1, 2, 3/1-2, 4/3/1-2, 5/4/3/1-2, 11-13, 14/11-13
A	WO 2012/004276 A2 (FONDAZIONE TELETHON et al.) January 12, 2012; entire document	1, 2, 3/1-2, 4/3/1-2, 5/4/3/1-2, 11-13, 14/11-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 January 2019 (02.01.2019)	22 JAN 2019
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/46861

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-10, 15-20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-***-Please See Within the Next Supplemental Box-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-5, 11-14; SEQ ID NO: 1

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/46861

-***-Continued from Box III: Lack of Unity of Invention-***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-5, 11-14 and SEQ ID NO: 1 are directed toward methods for determining a succination level of a protein in a test sample by determining the presence or quantity of modified cysteines in hydrolysates of protein from said sample.

The methods will be searched to the extent that they encompass a peptide of SEQ ID NO: 1 (first exemplary modified peptide). Applicant is invited to elect additional peptide(s), with specified SEQ ID NO: for each, such that the sequence of each elected species is fully specified (i.e. no optional or variable residues or substituents), to be searched. Additional peptide sequence(s) will be searched upon the payment of additional fees. It is believed that claims 1-5, 11, 12 (in part), 13 (in-part) and 14 (in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 1 (modified peptide). Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be SEQ ID NO: 2 (modified peptide).

No technical features are shared between the peptide sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features of: a method for determining a succination level in a test sample, comprising: hydrolyzing proteins of a test sample; determining the presence or quantity of modified cysteines in the hydrolyzed proteins, wherein the modified cysteines have been modified by one or more of dimethyl fumarate, monomethyl fumarate, or fumarate; and the method for determining a succination level in a test sample, comprising the preliminary steps of: isolating peripheral blood mononuclear cells of the sample; lysing the peripheral blood mononuclear cells; and enzymatically digesting proteins of the lysed peripheral blood mononuclear cells; however, these shared technical features are previously disclosed by the publication entitled, "Identification of Protein Succination as a Novel Modification of Tubulin," by Piroli et al. (hereinafter 'Piroli') in view of WO 2012/004276 A2 to Fondazione Telethon et al. (hereinafter 'Fondazione Telethon').

Piroli discloses a method for determining a succination level (determining relative abundance of succinated tubulin peptides; page 5, third paragraph) in a test sample (porcine brain tubulin sample; page 11, second paragraph), comprising: hydrolyzing proteins of a test sample (porcine brain tubulin cells hydrolyzed in 6M HCl; page 6, second paragraph); determining the presence or quantity of modified cysteines (determining DMF succination of tubulin cysteine residues; page 10, first paragraph) in the hydrolyzed proteins (porcine brain tubulin cells hydrolyzed in 6M HCl; page 6, second paragraph or trypsin digest peptides; page 5, third paragraph), wherein the modified cysteines have been modified by one or more of dimethyl fumarate (abstract; page 5, third paragraph), monomethyl fumarate, or fumarate. Piroli also discloses enzymatically digesting proteins (trypsin protein digests; page 5, third paragraph) of lysed cells (cell lysates; page 3, third paragraph), but Piroli does not disclose the method for determining a succination level in a test sample, comprising the preliminary steps of: isolating peripheral blood mononuclear cells of the sample; lysing the peripheral blood mononuclear cells; and enzymatically digesting proteins of the lysed peripheral blood mononuclear cells.

Fondazione Telethon discloses analyzing peripheral blood mononuclear cells (PBMC) (biomarker determination in PBMCs, page 1, lines 6-13) from isolated PBMCs (page 4, lines 1-5) using enzymatically digested protein samples (MS analysis of trypsin peptides; page 18, lines 18-27) to determine cysteine residue modifications (page 18, line 27 to page 19, line 1). It would have been obvious to one of ordinary skill in the art at the time of the invention to have modified the disclosure of Piroli to provide the preliminary steps of isolating peripheral blood mononuclear cells of the sample; lysing the peripheral blood mononuclear cells; and enzymatically digesting proteins of the lysed peripheral blood mononuclear cells in the method of determining a succination level in a test sample, because the ability to utilize lysates of peripheral blood mononuclear cells as a sample for the determination of cysteine modification as disclosed by Fondazione Telathon would have made peripheral blood mononuclear cells a suitable sample for the method of determining succination levels of cysteine in protein digests as previously disclosed by Piroli when the protein is obtained form said cells.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the combination of the Piroli and Fondazione Telethin references, unity of invention is lacking.