Identification of actin as a direct proteomic target of berberine, using an affinity-based chemical probe and elucidation of its modulatory role in actin assembly

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Experimental section

Chemistry

General methods. Unless otherwise described, all commercial reagents and solvents were purchased from commercial suppliers such as Sigma-Aldrich, and used without further purifications. Tetrahydrofuran was distilled from sodium benzophenone ketyl and dichloromethane, acetonitrile, triethylamine and pyridine were freshly distilled with calcium hydride. Flash column chromatography was carried out using silica-gel 60 (230-400 mesh, Merck). Thin layer chromatography was performed using 0.25 mm silica gel plates to monitor reactions. All reactions were performed under dry argon atmosphere in flame-dried glassware. $^1$H NMR and $^{13}$C NMR spectra were recorded on a BRUKER AVANCE 400 (400MHz), or VARIAN VNMRS 500 (500MHz) spectrometers, respectively. Chemical shifts are provided in parts per million (ppm, $\delta$) downfield from tetramethylsilane (internal standard) with coupling constant in hertz (Hz). Multiplicity is indicated by the following abbreviations: singlet (s), doublet (d), doublet of doublet (dd), triplet (t), quartet (q), quintet (quin) multiplet (m) and broad (br). Optical rotations were measured using JASCO DIP-1000 digital polarimeter at ambient temperature using 100 nm cell of 2 mL capacity. Mass spectra and HRMS were recorded on Agilent 6530 Accurate-Mass Q-TOF LC/MS and JEOL JMS-700. The purity of the compounds was identified by normal phase high-pressure liquid chromatography (HPLC) is carried out either on.

Compound (2) To a solution of 1 (1.006 mmol, ref: Anticancer Agents Med. Chem., 2015, 15, 89-98.) in DMF (10 mL) was added propargyl bromide (2.415 mmol). The reaction mixture was stirred at 80 °C until complete consumption of the starting material on TLC. The reaction mixture was recrystallized from diethyl ether to afford 398mg (100%) of compound 2 as a brown solid. $^1$H-NMR (DMSO, 300 MHz) $\delta$ 9.88 (s, 1H), 8.97 (d, 1H, $J$ = 7.8 Hz), 8.22 (t, 1H, $J$ = 8.8 Hz), 8.03 (dd, 1H, $J$ = 14.8, 9.1 Hz), 7.80 (s, 1H), 7.09 (s, 1H), 6.18 (s, 2H), 5.09 (d, 2H, $J$ = 2.4 Hz), 4.96 (t, 2H, $J$ = 6.1 Hz), 4.09 (d, 3H, $J$ = 5.6 Hz), 3.62 (t, 1H, $J$ = 2.4 Hz), 3.21 (t, 2H, $J$ = 6.1 Hz); $^{13}$C-NMR (DMSO, 75 MHz) $\delta$ 151.2, 150.4, 148.2, 145.8, 141.2, 138.1, 133.4, 131.2, 127.0, 124.8, 122.6, 120.9, 120.8, 108.9, 106.0, 102.6, 80.3, 79.3, 61.4, 57.7, 55.8, 26.8; LR-MS (FAB) m/z 360 (M-Cl)$^+$; HR-MS (FAB) calculated for C$_{22}$H$_{18}$NO$_4^+$ (M-Cl)$^+$ 360.1236; found 360.1237.

Compound (4) To a solution of 2 (0.253 mmol), and $N$-[2-[2-(2-azidoethoxy)ethoxy]-ethoxy]ethyl]-biotinamide (3, 0.253 mmol) in $t$-BuOH (3 mL) and H$_2$O (3 mL) were added sodium ascorbate (0.487 mmol) and copper(II) sulfate (0.152 mmol). The reaction mixture was stirred at room temperature until complete consumption of the starting material on TLC. Then, the solvent was concentrated in vacuo and purified by column chromatography on silica gel (methylene chloride:
methanol: water = 70:14:1) to afford 60 mg (28%) of compound 3 as a yellow solid; $^1$H-NMR (MeOD, 400 MHz) $\delta$ 9.63 (s, 1H), 8.68 (s, 1H), 8.21 (s, 1H), 8.12 (d, 1H, $J = 9.2$ Hz), 8.01 (d, 1H, $J = 9.1$ Hz), 7.63 (s, 1H), 6.95 (s, 1H), 6.10 (s, 2H), 5.55 (s, 2H), 4.91 (t, 2H, $J = 6.3$ Hz), 4.57 (t, 2H, $J = 5.0$ Hz), 4.47 (dd, 1H, $J = 7.8$, 4.8 Hz), 4.27 (dd, 1H, $J = 7.9$, 4.5 Hz), 4.15 (s, 3H), 3.85 (t, 2H, $J = 5.0$ Hz), 3.56-3.53 (m, 8H), 3.49 (t, 2H, $J = 5.7$ Hz), 3.31-3.29 (m, 2H), 3.25 (t, 2H, $J = 6.6$ Hz), 3.19-3.14 (m, 1H), 2.89 (m, 1H), 2.67 (d, 1H, $J = 12.7$ Hz), 2.17-2.13 (m, 2H), 1.67-1.29 (m, 6H); $^{13}$C-NMR (MeOD, 125 MHz) $\delta$ 174.9, 164.9, 151.4, 151.0, 148.7, 145.2, 142.3, 142.1, 138.5, 133.9, 130.8, 126.4, 125.9, 124.1, 122.7, 120.6, 120.4, 108.3, 105.4, 102.5, 70.4, 70.3, 70.1, 69.4, 69.1, 65.9, 62.2, 60.4, 56.5, 56.1, 55.8, 53.7, 50.2, 39.9, 39.1, 35.5, 28.6, 28.3, 27.0, 25.7; LR-MS (ESI) m/z 804 (M-Cl)$^+$; HR-MS (ESI) calculated for C$_{40}$H$_{50}$N$_7$O$_9$S$^+$ (M-Cl)$^+$ 804.3385; found 804.3377.
Compound 4 (BBP)

\[ ^1\text{H-NMR (CDCl}_3, 400 \text{ MHz)} \]
\[ ^{13}\text{C-NMR (CDCl}_3, 125\text{ MHz)} \]
Biological assay

Pull-down assay

Jurkat human leukemic T-cells (ATCC TIB-152) were maintained in RPMI medium containing 10% FBS and penicillin/streptomycin (100 U/ml). Jurkat cells were lysed with lysis buffer (XX mM Tris-HCl containing 0.25% NP-40 and protease inhibitor cocktail). The lysates (10 mg) were incubated with Biotin-azide (20 μM) or Berberine biotinylated probe (BBP) (20 μM) with or without free berberine (40 μM) for 2 hours, followed by further incubation with streptavidin-resin for 30 minutes. After extensive washing with lysis buffer 5 times, BBP-bound proteins were eluted with SDS-PAGE sample buffer and subjected to SDS-PAGE and silver staining according to the manufacturer’s instruction (Pierce). The band was excised and analyzed using LC-MS/MS by Yonsei Proteome Research Center (YPRC).

LC-MS/MS for peptide analysis

NanoLC–MS/MS analysis was performed on an agilent 1100 Series nano-LC and LTQ- mass spectrometer (Thermo Electron, San Jose, CA). The capillary column used for LC–MS/MS analysis (150 mm × 0.075 mm) was obtained from Proxeon (Odense M, Denmark) and slurry packed in house with 5 μm, 100 Å pore size Magic C18 stationary phase (Michrom Bioresources, Auburn, CA). The mobile phase A for the LC separation was 0.1% formic acid in deionized water and the mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was set up to give a linear increase from 6 % B to 50 % B in 17 min and from 50 % B to 95 % B in 6 min and from 95 % B to 6 % B in 12 min. The flow rate was maintained at 600 nL/min after splitting. Mass spectra were acquired using data-dependent acquisition with full mass scan (350-1800 m/z) followed by MS/MS scans. Each MS/MS scan acquired was an average of one microscans on the LTQ. The temperature of the ion transfer tube was controlled at 200 °C and the spray was 1.5.0-2.0 kV. The normalized collision energy was set at 35% for MS/MS.

Database searching

The mascot algorithm (Matrixscience, USA) was used to identify peptide sequences present in a protein sequence database. Database search criteria were, taxonomy; homo sapiens (NCBI nr database downloaded on Mar 24 2013; 695124 sequences: 228548881 residues), fixed modification; carboxyamidomethylated at cysteine residues, variable modification; oxidized at methionine residues, maximum allowed missed cleavage: 2, MS tolerance: 1.2 Da, MS/MS tolerance: 0.6 Da. Only peptides resulting from trypsin digests were considered.
**Direct binding assay**

Purified rabbit actin (13.5 μg) was reacted with BBP (20 μM) with or without free berberine (BBR, 40 μM) for 2 hours followed by dialysis using Tris-HCl (pH 8.0) buffer to remove free BBP. The samples were further incubated with streptavidin resin for 30 minutes and washed with Tris-HCl buffer five times. Precipitated samples were subjected to SDS-PAGE and silver staining (Pierce) according to the instruction. To further confirm the interaction between berberine and actin Surface Plasmon Resonance (SPR) assay was carried out.

**Podosome reformation assay**

Raw 264.7 cells (ATCC TIB-71) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS and penicillin/streptomycin (100 U/ml). Cells were starved for 24 hours with serum-free DMEM, then treated with 25 μM PP2 for 30 minutes to remove podosomes. After podosome disruption, cells were washed with phosphate buffered saline (PBS) and fed with normal complete medium containing DMSO or BBR (100 μM). After 30 minutes of incubation, cells were washed once with PBS and then fixed with 4% paraformaldehyde for an hour. Fixed cells were washed with PBS once and permeabilized with 0.1% Triton-X100 for 15 min. After permeabilization, cells were washed two or more times with PBS and stained with CytoPainter F-actin staining kit-Green Fluorescence (Abcam) for an hour to visualize newly formed podosomes.

**Actin polymerization and depolymerization Assay**

*In vitro* actin polymerization and depolymerization assays were carried out using fluorescence-based actin polymerization kit (Cytoskeleton, Cat. No. BK003) according to the manufacturer’s instruction. Briefly, polymerization buffer (10X) was added to prepared pyrene-G-actin samples with indicated concentrations of free berberine. Actin polymerization was assessed by monitoring fluorescence signal using a fluorimeter (Tecan). To assess the depolymerizing activity of berberine, pyrene F-actin was prepared by polymerization of G-actin for 1 hour according to the instruction. Indicated concentrations of berberine were added to the samples and depolymerization was monitored for 90 minutes using a fluorimeter.

**Imaging of actin filaments after the treatment of berberine.**

NIH3T3 cell line was cultured in DMEM supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, 1 U/ml penicillin, 100 mg/ml streptomycin, and 1 mM sodium pyruvate. For imaging experiment, cells were plated onto 35 mm glass coverslip-bottomed dishes coated with 10 μg/ml of
fibronectin (Invitrogen). Cells were washed once with pre-chilled phosphate buffered saline (PBS) and incubated with PBS containing 25 μl/ml 0.1% Triton-X100 for 10 min at 4°C. Then, the cells were washed once with pre-warmed media and returned to the incubator with culture media with or without 100 μM berberine chloride. After 48 hours of incubation, cells were washed once with pre-warmed PBS and then fixed with 4% paraformaldehyde. Fixed cells were washed two or more times with PBS for 10 min and permeabilized with pre-chilled 0.1% Triton-X100 for 5 min, after washed again and then, blocked with 1% BSA (company) for 30 minutes in room temperature. After blocking, cells were washed two or more times with PBS for 10 minutes and stained with PBS containing Alexa Fluor 594 Phalloidin (1:200)(Life technology) for 30 minutes. Images were collected by a Nikon Ti-E inverted microscope and a cooled charge-coupled device camera using NIS software with a 560DF40 excitation filter, a 595DRLP dichroic mirror, and a 653DF95 emission filter. A neutral-density filter was used to control the intensity of the excitation light. Collected images were analyzed with NIS software.
Supplementary Figure S1. LC-MS/MS analyses of a tryptic digest of the protein band pulled-down by BBP.
Peptide View
MS/MS Fragmentation of IKIIAPPERS
Found in gi|28252 in NCBInr_human, unnamed protein product [Homo sapiens]
Match to Query 926: 1036.428090 from(519.221436,2+)
Title: spectrumId=1481 Filter=ITMS + c NSI d Full ms2 519.22@cid35.00 [130.00-1050.00] PeakProcessing=continuous Polarity=positive
ScanMode=MassScan TimeInMinutes=10.225383 acqNumber=1481

Average mass of neutral peptide Mr(calc): 1036.2663 Fixed modifications:
Carbamidomethyl (C) (apply to specified residues or termini only) Ions Score: 32
Expect: 3 Matches : 8/78 fragment ions using 16 most intense peaks

Peptide View
MS/MS Fragmentation of GYSFTTAER
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Match to Query 1094: 1132.206776 from(567.110779,2+)
Title: spectrumId=1557 Filter=ITMS + c NSI d Full ms2 567.11@cid35.00 [145.00-1145.00] PeakProcessing=continuous Polarity=positive
ScanMode=MassScan TimeInMinutes=11.186387 acqNumber=1557

Average mass of neutral peptide Mr(calc): 1132.1803 Fixed modifications:
Carbamidomethyl (C) (apply to specified residues or termini only) Ions Score: 53
Expect: 0.023 Matches : 14/84 fragment ions using 26 most intense peaks
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Expect: 2.5e+002 Matches: 4/88 fragment ions using 7 most intense peaks

Peptide View
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Average mass of neutral peptide Mr(calc): 1198.4173 Fixed modifications: Carboxymethyl (C) (apply to specified residues or termini only) Ions Score: 21 Expect: 28 Matches : 8/84 fragment ions using 7 most intense peaks
Peptide View
MS/MS Fragmentation of AVFPSIVGRPR
Found in gi|28252 in NCBI1r_human, unnamed protein product [Homo sapiens]
Match to Query 1188: 1198.806384 from(600.410583)2+
Title: spectrumId=1642 Filter=ITMS + c NSI d Full ms2 600.41@cid35.00 [155.00-1215.00] PeakProcessing=continuous Polarity=positive
ScanMode=MassScan TimeInMinutes=12.231093 accumber=1642

Average mass of neutral peptide Mr(calc): 1198.4173 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only) Ions Score: 52 Expect: 0.026 Matches : 30/84 fragment ions using 73 most intense peaks

Peptide View
MS/MS Fragmentation of AVFPSIVGRPR
Found in gi|28252 in NCBI1r_human, unnamed protein product [Homo sapiens]
Match to Query 1189: 1199.478382 from(600.746582)2+
Title: spectrumId=1661 Filter=ITMS + c NSI d Full ms2 600.75@cid35.00 [155.00-1215.00] PeakProcessing=continuous Polarity=positive
ScanMode=MassScan TimeInMinutes=12.472018 accumber=1661

Average mass of neutral peptide Mr(calc): 1198.4173 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only) Ions Score: 61 Expect: 0.0047 Matches : 29/84 fragment ions using 56 most intense peaks
Peptide View
MS/MS Fragmentation of EITALAPSTMKIK
Found in g|28252 in NCBInr_human, unnamed protein product [Homo sapiens]
Match to Query 834: 1419.749526 from(474.257233,3+ )
Title: spectrumld=1490 Filter=1ITMS + c NSI d Full ms2 474.26@cid35.00 [120.00-960.00] PeakProcessing=continuous Polarity=positive
ScanMode=MassScan TimeInMinutes=10.338302 acqNumber=1490

| b  | b** | b*** | b**** | b***** | Seq | y    | y**   | y***  | y**** | y***** | y****** | y******* | M   |
|----|-----|------|-------|--------|-----|------|-------|-------|-------|--------|---------|----------|------|-----|
| 1  | 130.1214 | 65.5644 | 112.1061 | 56.5566 | E | 1250.3917 | 645.7995 | 1273.5512 | 637.2845 | 1272.5762 | 636.7919 | 12 |
| 2  | 243.2793 | 122.1432 | 225.2567 | 113.1330 | I | 1297.5434 | 650.8381 | 1305.5800 | 641.6540 | 1297.5434 | 640.8381 | 12 |
| 3  | 344.3829 | 167.6951 | 326.0266 | 163.0167 | T | 1317.4414 | 659.0590 | 1317.4414 | 659.0590 | 1317.4414 | 659.0590 | 11 |
| 4  | 415.4605 | 208.2413 | 397.4485 | 208.2413 | I | 1353.6302 | 693.1265 | 1353.6302 | 693.1265 | 1353.6302 | 693.1265 | 10 |
| 5  | 528.6163 | 244.6179 | 501.5319 | 244.6179 | L | 1401.7232 | 733.2836 | 1401.7232 | 733.2836 | 1401.7232 | 733.2836 | 10 |
| 6  | 599.6962 | 300.3518 | 581.6581 | 300.3518 | I | 1434.8047 | 766.5150 | 1434.8047 | 766.5150 | 1434.8047 | 766.5150 | 9 |
| 7  | 696.8115 | 348.9094 | 678.7962 | 348.9094 | P | 1521.9168 | 811.0121 | 1521.9168 | 811.0121 | 1521.9168 | 811.0121 | 9 |
| 8  | 783.8883 | 392.4481 |                   |                   |          |                   |                   |                   |                   |                   |                   | 7 |

Average mass of neutral peptide Mr(calc): 1418.6982 Fixed modifications: Carboxymethyl (C) (apply to specified residues or termini only)
Variable modifications: M10 : Oxidation (M), with neutral losses 0.0000(shown in table), 64.1069 Ions Score: 17 Expect: 96 Matches : 19/184 fragment ions using 40 most intense peaks

Peptide View
MS/MS Fragmentation of QEYDESGPSIVHR
Found in g|28252 in NCBInr_human, unnamed protein product [Homo sapiens]
Match to Query 898: 1517.233982 from(506.752045,3+ )
Title: spectrumld=1425 Filter=1ITMS + c NSI d Full ms2 506.75@cid35.00 [125.00-1025.00] PeakProcessing=continuous Polarity=positive
ScanMode=MassScan TimeInMinutes=0.510245 acqNumber=1425

| a  | b  | b** | b*** | b**** | Seq | y    | y**   | y***  | y**** | y***** | y****** | y******* | M   |
|----|----|-----|------|-------|-----|------|-------|-------|-------|--------|---------|----------|------|-----|
| 1  | 129.1364 | 65.6702 | 120.1061 | 56.5566 | O | 1389.4462 | 695.2845 | 1372.4571 | 686.7115 | 1371.4309 | 686.2819 | 12 |
| 2  | 298.2689 | 129.6296 | 241.2401 | 121.1137 | G | 1420.2320 | 720.5880 | 1402.2320 | 720.5880 | 1402.2320 | 720.5880 | 12 |
| 3  | 421.4373 | 211.2158 | 340.2686 | 202.0060 | D | 1451.4373 | 754.0320 | 1433.4373 | 744.0320 | 1433.4373 | 744.0320 | 12 |
| 4  | 536.5112 | 265.7575 | 439.5939 | 265.7575 | E | 1482.5225 | 788.0725 | 1464.5225 | 778.0725 | 1464.5225 | 778.0725 | 12 |
| 5  | 659.6254 | 323.1302 | 535.1726 | 323.1302 | Q | 1513.6179 | 821.1679 | 1495.6179 | 811.1679 | 1495.6179 | 811.1679 | 12 |
| 6  | 752.7603 | 376.8550 | 628.4783 | 376.8550 | N | 1544.7526 | 855.3226 | 1526.7526 | 845.3226 | 1526.7526 | 845.3226 | 12 |
| 7  | 809.8703 | 424.4058 | 679.5214 | 424.4058 | R | 1577.8602 | 889.3802 | 1559.8602 | 879.3802 | 1559.8602 | 879.3802 | 12 |
| 8  | 916.9660 | 478.5032 | 756.1504 | 478.5032 | L | 1608.9560 | 932.9360 | 1590.9560 | 922.9360 | 1590.9560 | 922.9360 | 12 |
| 9  | 1023.0614 | 532.6007 | 887.2165 | 532.6007 | Y | 1640.0561 | 987.4961 | 1622.0561 | 977.4961 | 1622.0561 | 977.4961 | 12 |
| 10 | 1129.1565 | 586.7002 | 951.2525 | 586.7002 | Y | 1723.1525 | 1042.0425 | 1705.1525 | 1032.0425 | 1705.1525 | 1032.0425 | 12 |
| 11 | 1236.2516 | 640.7998 | 1015.3038 | 640.7998 | Q | 1815.2475 | 1100.1375 | 1797.2475 | 1090.1375 | 1797.2475 | 1090.1375 | 12 |
| 12 | 1343.3467 | 694.9004 | 1089.3927 | 694.9004 | Y | 1910.3387 | 1155.1287 | 1892.3387 | 1145.1287 | 1892.3387 | 1145.1287 | 12 |

Average mass of neutral peptide Mr(calc): 1516.5879 Fixed modifications: Carboxymethyl (C) (apply to specified residues or termini only) Ions Score: 64 Expect: 0.0015 Matches : 37/134 fragment ions using 54 most intense peaks
Peptide View
MS/MS Fragmentation of MQKEITALAPSTMK
Found in gi|28252 in NCBIInr_human, unnamed protein product [Homo sapiens]
Match to Query 965: 1581.262740 from(528.094971,3+)
Title: spectrumId=1457 Filter=ITMS + c NSI d Full ms2 528.09@cid35.00 [135.00-1070.00] PeakProcessing=continuous Polarity=positive
ScanMode=MassScan TimeInMinutes=9.924432 acqNumber=1457

Average mass of neutral peptide Mr(calc): 1580.8854 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only) Variable modifications: M1 : Oxidation (M), with neutral losses 0.0000(shown in table), 64.1069 M13 : Oxidation (M), with neutral losses 0.0000(shown in table), 64.1069 Ions Score: 2 Expect: 2.7e-003 Matches : 15/264 fragment ions using 23 most intense peaks

Peptide View
MS/MS Fragmentation of MQKEITALAPSTMK
Found in gi|28252 in NCBIInr_human, unnamed protein product [Homo sapiens]
Match to Query 966: 1581.494184 from(528.172119,3+)
Title: spectrumId=1393 Filter=ITMS + c NSI d Full ms2 528.17@cid35.00 [135.00-1070.00] PeakProcessing=continuous Polarity=positive
ScanMode=MassScan TimeInMinutes=9.098173 acqNumber=1393

Average mass of neutral peptide Mr(calc): 1580.8854 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only) Variable modifications: M1 : Oxidation (M), with neutral losses 0.0000(shown in table), 64.1069 M13 : Oxidation (M), with neutral losses 0.0000(shown in table), 64.1069 Ions Score: 45 Expect: 0.15 Matches : 39/264 fragment ions using 40 most intense peaks
Peptide View
MS/MS Fragmentation of SYELPDGQVTIGNER
Found in gi|1627852 in NCBI_nr_human, unnamed protein product [Homo sapiens]
Match to Query 1724: 1790.426624 from(896.220703,2+)
Title: spectrumId=1915 Filter=ITMS + c NSI d Full ms2 896.22@cid35.00 [235.00-1805.00] PeakProcessing=continuous Polarity=positive
ScanMode=MassScan TimeInMinutes=15.703047 accNumber=1915

Average mass of neutral peptide Mr(calc): 1790.9243 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only) Ions Score: 58 Expect: 0.0048 Matches: 31/164 fragment ions using 50 most intense peaks

Peptide View
MS/MS Fragmentation of SYELPDGQVTIGNER
Found in gi|1627852 in NCBI_nr_human, unnamed protein product [Homo sapiens]
Match to Query 1724: 1790.426624 from(896.220703,2+)
Title: spectrumId=1915 Filter=ITMS + c NSI d Full ms2 896.22@cid35.00 [235.00-1805.00] PeakProcessing=continuous Polarity=positive
ScanMode=MassScan TimeInMinutes=15.703047 accNumber=1915

Average mass of neutral peptide Mr(calc): 1790.9243 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only) Ions Score: 84 Expect: 1.1e-005 Matches: 42/164 fragment ions using 62 most intense peaks
Peptide View
MS/MS Fragmentation of MQKEITALAPSTMKIK
Found in gi|28252 in NCBI.nr_human, unnamed protein product [Homo sapiens]
Match to Query 1212: 1822.094769 from(608.372314,3+)
Title: spectrumId=1488 Filter=ITMS + c NSI d Full ms2 608.37@cid35.00 [155.00-1230.00] PeakProcessing=continuous Polarity=positive ScanMode=MassScan TimeInMinutes=10.319560 acqNumber=1488

Average mass of neutral peptide Mr(calc): 1822.1953 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only) Variable modifications: M1 : Oxidation (M), with neutral losses 0.00000(shown in table), 64.1069 M13 : Oxidation (M), with neutral losses 0.00000(shown in table), 64.1069 Ions Score: 15 Expect: 1.4e+002 Matches: 41/316 fragment ions using 76 most intense peaks

Peptide View
MS/MS Fragmentation of VAPEEHVPLLTEAPLNPK
Found in gi|28252 in NCBI.nr_human, unnamed protein product [Homo sapiens]
Match to Query 1759: 1953.677844 from(977.846313,2+)
Title: spectrumId=1692 Filter=ITMS + c NSI d Full ms2 977.85@cid35.00 [255.00-1970.00] PeakProcessing=continuous Polarity=positive ScanMode=MassScan TimeInMinutes=12.864683 acqNumber=1692

Average mass of neutral peptide Mr(calc): 1954.2269 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only) Ions Score: 41 Expect: 0.25 Matches: 35/156 fragment ions using 90 most intense peaks
**Peptide View**
MS/MS Fragmentation of VAPEELPVLEELTEAPLNPK
Found in gi|28252 in NCBI-nr: human, unnamed protein product [Homo sapiens]
Match to Query 1342: 1055.121281 from(652.714478,3+)
Title: spectrumId=1732 Filter=ITMS + c NSI d Full ms2 652.71@cid35.00 [165.00-1320.00] PeakProcessing=continuous Polarity=positive
ScanMode=MassScan TimeInMinutes=13.388082 acqNumber=1732

Average mass of neutral peptide Mr(calc): 1954.2269 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only) Ions Score: 36
Expect: 1.3 Matches : 34/156 fragment ions using 73 most intense peaks

**Peptide View**
MS/MS Fragmentation of VAPEELPVLEELTEAPLNPK
Found in gi|28252 in NCBI-nr: human, unnamed protein product [Homo sapiens]
Match to Query 1344: 1955.350509 from(652.790894,3+)
Title: spectrumId=1690 Filter=ITMS + c NSI d Full ms2 652.79@cid35.00 [165.00-1320.00] PeakProcessing=continuous Polarity=positive
ScanMode=MassScan TimeInMinutes=12.834872 acqNumber=1690

Average mass of neutral peptide Mr(calc): 1954.2269 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only) Ions Score: 36
Expect: 1.3 Matches : 41/156 fragment ions using 102 most intense peaks
Peptide View
MS/MS Fragmentation of IKIIAPPER
Found in gi|28252 in NCBIr_human, unnamed protein product [Homo sapiens]
Match to Query 926: 1036.428090 from(519.221436,2+)
Title: spectrumId=1481 Filter=ITMS + c NSI d Full ms2 519.22@cid35.00 [130.00-1050.00] PeakProcessing=continuous Polarity=positive
ScanMode=MassScan TimeInMinutes=10.225383 acqNumber=1481

Average mass of neutral peptide Mr(calc): 1954.2269 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only) Ions Score: 39 Expect: 0.61 Matches: 46/156 fragment ions using 109 most intense peaks

Peptide View
MS/MS Fragmentation of VAPEEHFVLLTEAPLNPK
Found in gi|28252 in NCBIr_human, unnamed protein product [Homo sapiens]
Match to Query 1346: 1955.356734 from(652.792969,3+)
Title: spectrumId=1755 Filter=ITMS + c NSI d Full ms2 652.79@cid35.00 [165.00-1320.00] PeakProcessing=continuous Polarity=positive
ScanMode=MassScan TimeInMinutes=13.651792 acqNumber=1755

Average mass of neutral peptide Mr(calc): 1954.2269 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only) Ions Score: 24 Expect: 21 Matches: 22/156 fragment ions using 44 most intense peaks
**Peptide View**

MS/MS Fragmentation of AGFAGDDAPRAVFSIVGRPR

Found in gi|28252 in NCBlnr_human, unnamed protein product [Homo sapiens]

Match to Query 1531: 2156.002059 from(719.674744,3+)

Title: spectrumID=1771 Filter=ITMS + c NSId Full ms2 719.67@cid35.00 [185.00-1450.00] PeakProcessing=continuous Polarity=positive ScanMode=MassScan TimeInMinutes=13.855678 acqNumber=1771

Average mass of neutral peptide Mr(calc): 2156.4032 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only) Ions Score: 55 Expect: 0.011 Matches : 48/200 fragment ions using 83 most intense peaks

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**Peptide View**

MS/MS Fragmentation of VAPEEHPVLLTEAPLPKANR

Found in gi|28252 in NCBlnr_human, unnamed protein product [Homo sapiens]

Match to Query 1596: 2294.828229 from(765.959034,3+)

Title: spectrumID=1631 Filter=ITMS + c NSId Full ms2 765.95@cid35.00 [200.00-1545.00] PeakProcessing=continuous Polarity=positive ScanMode=MassScan TimeInMinutes=12.094430 acqNumber=1631

Average mass of neutral peptide Mr(calc): 2295.5931 Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only) Ions Score: 20 Expect: 46 Matches : 20/186 fragment ions using 55 most intense peaks