Human Insulin

MW 5808

Introduction

- Cyclic peptide (CD) has better biological activity compared to their linear due to counterparts the conformational rigidity.
- Resistance to hydrolysis due to the lack of both amino and carboxyl termini.
- Some cyclic peptides can cross the cell membrane
- 9 Cyclic peptide drugs have been approved by FDA and/or EMA from 2006 to 2014

Challenges in identifying cyclic peptide metabolites by LC/MS

- Commonly employed LC/MS data processing tools for finding metabolites of small molecule drugs are not well suited for analysis CP metabolites
- CPs often contain unnatural amino acids and peptide linkages
- CP metabolites are difficult to generate high quality and interpretable fragments due to their unique structures (multiple disulphide linkages)
- The number of theoretical metabolite products of a CP drug is huge, searching for all of predicted metabolites is impractical

Objective

• Apply and test recently expanded tool, MetabolitePilot[™] software, for automated detection and structural characterization of cyclic peptide metabolites using insulin as model cyclic peptide

Experimental conditions

- Insulin was incubated with Trypsin and Chymotrypsin in buffer for 0 to 3h
- Chromatography separation was performed on a Waters Acquity BEH C18 column (1.7 um, 2.1 x 100 mm) using mobile phases 0.1% formic acid in water and 0.1% formic acid in acetonitrile.
- High-resolution mass spectrometry data were obtained using the TripleTOF® 5600 system (SCIEX) with IDA acquisitions (CE of 50.0). IDA for MS/MS only include the charge state from 2 to 5, and up to 20 MS/MS spectra with dynamic exclusion.
- The data were analyzed with MetabolitePilot[™] software 2.0 based on an insulin-tailored processing method using the TOF-MS predicted metabolite peak finder, multiple- charge filter and TOF-MSMS characteristic product ions peak finder.

Workflow for detecting and identifying metabolites of cyclic peptides using MetabolitePilotTM (Sciex)



TOF MS

Predicted metabolites

Generic peak finding

Mass defect

TOF MSMS

Isotope pattern

 All specified ions At least 2 ions

All specified losses

At least 1 losses

Apply mass defect filter

Apply charge state filter

Find characteristic product ions

Find characteristic neutral losses

Consider internal neutral losses

Isotope pattern (SWATH® Only)

Peak finding strategy from MS datasets

- >Predicted metabolites strategy finds peaks in accurate mass XICs for parent compound and predicted hydrolytic cleavage products with or without additional biotransformation.
- >Generic peak finder strategy finds 3-dimensional peaks in the LC/MS data, then removes entries that do not have charge state within the user selected range.
- \succ If any control samples are used in processing, peaks that are present in control samples are filtered from the results.
- \rightarrow Accurate m/z and peak charge state are confirmed in the TOF MS spectra.

Fast and comprehensive detection and characterization of cyclic peptide metabolites using software-aided data processing tools

Ming Yao¹, Eva Duchoslav², <u>Li Ma¹</u>, Silvi Chacko¹, Mingshe Zhu^{1,3} ¹Pharmaceutical Candidate Characterization, Bristol-Myers Squibb, Princeton, NJ 08543, USA. ²Sciex, Concord, Ontario, L4K 4V8, CANADA. ³Current Address: MassDefect Technologies, Princeton, NJ 08540 USA



Insulin fragments

Workspace	Oper	n	Save	Save As View Show: O Results O In	terpretation	
	Pot	ential	l Metabolit	tes: 57 of 57 Peaks		
Datab		Rep	ort Peak IE	Name		Formula
Batch	50			Parent [M+6H]6+	C257	H383N6
Sk	51		M20-2	2 GIVEQC[*1]C[*2]TSIC[*1]SLYQLENYCN / FVNQHLC[*2]GS+Methylatic	on [M+3H]3+ C143	H218N3
- * <	52			Parent [M+5H]5+	C257	H383N6
Results	53		M14	GFFYTPKT [M+2H]2+	C48H	65N9O1
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Workflow			129.1022 √			
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519.5699 (7.7 ppm, C63H104N19O23S2+++) [M+3H]3+

515 516 517 518 519 520 521 522 523 524

m/z, Da

520.9042

+TOF MS at 12.43 min

161 of both singly and multiply charged fragments (~70% TIC) could be associated with insulin sequence. Due to the insulin structure larger fragments had relatively low abundance but they maintained good mass accuracy.

Processing method for searching for peptide metabolites eneric Parameters Compound-Specific Parameters

510.156

511.275

519.236

520.206

Biotransform Use this set: Biotransforma	nations parent only biologics itions selected: 9			Select Se
	Name		Mass Shift	Description
Loss of Wate	r		-18.0106	R-H2O to R
Demethylatio	n		-14.0157	R-CH3 to R-H
Desaturation			-2.0157	R-CH2-CH2-R1 to R-CHCH-R1
Parent			0.0000	Parent (P)
Demethylatio	n and Oxidation		1.9792	R-CH3 to R-OH
Hydrogenatio	n		2.0157	+2H
Methylation			14.0157	R-H to R-CH3
Oxidation			15.9949	+0
Internet I bude	-hu-i-		12 0106	

Close

Identification of insulin metabolites in enzymatic incubation (1h)

pretation									How Do	o 1?	Х
Formula	Assisted	Neutral M	Y Assign ID Add <u>MS/MS</u>				Momo	Analog In	egration		
146H223N37O4	Assigned	3342 53	836 6388	4	F	2 4	25.35	1 07E+07	21.63	57.7	Ē
63H101N19O23S2		1555.69	519 5699	3		7.7	12.43	6.45E+06	13.00	26.3	
44H58N8O10		858.43	430.2247	2		8.5	17.65	9,40E+05	1.89	31.9	
48H65N9O12	√	959.48	480.7492	2		9.0	18.21	8.15E+05	1.64	41.1	_
257H383N65O7	1	5803.62	1161.7320	5	3	-2.4	27.28	5.96E+05	1.20	54.8	
63H99N19O22S2	1	1537.67	769.8426	2		3.4	15.54	5.29E+05	1.07	44.4	
67H98N18O20S2	~	1538.66	770.3349	2		-6.1	16.08	4.92E+05	0.99	32.6	
28H44N6O9		608.32	305.1688	2		9.9	5.37	4.26E+05	0.86	30.8	
37H53N7O10	~	755.39	378.7036	2		9.5	11.78	3.21E+05	0.65	38.0	
238H350N60O7		5376.40	1076.2866	5	2	0.4	27.97	2.75E+05	0.55	60.5	_
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es 🔽									S	how controls	
25 Show controls											
12	2.43 (M4)							N			
	12.91	(M5)	20.20	(M4.2)				25.35 (M2	1, M22, M	23)	
1 (M2) 11.78 (M3)		15.54 (M7)	17.65 (M10)	(M13) 2	4.86 (N	117, M18	, M19, M20)	27.28 (Pa	rent, M27)	
9 10 11	12 13	14 15 16	17 18	19 20	° 21	22 2	23 24 2	26°26 27	28	29 30 3	1
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m/z, Da								m/z, Da			

Identification of M4, [,] a major metabolite of Insulin in enzymatic incubation







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M7 (N
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N
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M18 (N
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1

The major enzymatic incubation products have peak areas shaded with dark blue. Details on the disulphide bonds in metabolite sequences are provided with the '[*x]' symbol, chains are separated with '/' symbol.

Major insulin catabolites found in enzymatic incubation (1h)

Insulin sequence: 51 AA Avg. Mol Wt: 5807.7, Accurate mass: 5803.67 Formula: C257H383N65O77S6



- challenges
- peptide spectral interpretation tools

- William Humphreys

characterized in enzymatic incubation (1 h)

eak ID	Name	Formula	Neutral Mass	m/z	Charge	R.Time (min)	Peak Area	MS/MS Assigned
M1	YTPKT	C28H44N6O9	608.32	305.1688	2	5.4	4.26E+05	٧
M2	QLENYC[*3]N/VC[*3]GER	C57H90N18O22S2	1442.60	722.3065	2	9.8	1.71E+04	
M3	FYTPKT	C37H53N7O10	755.39	378.7036	3	11.8	3.21E+05	٧
M4	QLENYC[*3]N/LVC[*3]GER	C63H101N19O23S2	1555.69	519.5699	2	12.4	6.45E+06	٧
M5	GIVEQC[*1]CT / IC[*1]SLYQ+Loss of Water	C65H104N16O22S3	1556.66	779.3393	2	12.9	5.19E+04	
M6	QLENYC[*3]N/YLVC[*3]GER	C72H110N20O25S2	1718.75	573.9243	3	14.9	1.62E+05	V
M4-H2O)	QLENYC[*3]N / LVC[*3]GER+Loss of Water	C63H99N19O22S2	1537.67	769.8426	2	15.5	5.29E+05	٧
M8	NYC[*3]N / LVC[*3]GERGFF+Hydrogenation	C67H98N18O20S2	1538.66	770.3349	2	16.1	4.92E+05	V
M9	QLENYC[*3]N/LVC[*3]GERGF	C74H113N21O25S2	1759.77	880.8916	2	16.4	5.28E+04	
M10	GFFYTPK	C44H58N8O10	858.43	430.2247	2	17.7	9.40E+05	٧
M11	GFFYTPKT	C48H65N9O12	959.48	480.7492	2	18.2	8.15E+05	٧
M12	SLYQLENYC[*3]N/LYLVC[*3]G	C85H127N19O27S2	1909.86	637.6257	3	19.4	2.54E+04	٧
M13	QLENYC[*3]N/LVC[*3]GERGFF	C83H122N22O26S2	1906.84	954.4265	2	20.3	8.16E+04	٧
M14	GIVEQC[*1]C[*2]TSIC[*1]SL/LC[*2]GSHLVE+Loss of Water	C90H148N24O31S4	2188.97	730.6647	3	21.0	2.27E+04	٧
M15	SLYQLENYC[*3]N/LYLVC[*3]GERGFFY	C125H176N28O36S2	2709.24	678.3180	4	21.2	1.46E+04	٧
M16	GIVEQC[*1]C[*2]TSIC[*1]SLY/FVNQHLC[*2]GSHL	C118H182N32O37S4	2767.23	692.8140	4	22.4	5.88E+04	٧
M17	GIVEQC[*1]C[*2]TSIC[*1]SL/FVNQHLC[*2]GSHL	C109H173N31O35S4	2604.14	869.0542	3	24.9	2.47E+05	٧
M24+H2O)	GIVEQC[*1]C[*2]TSI / C[*1]SLYQLENYC[*3]N / FVNQHLC[*2]GSHLVEALYLVC[*3]GER	C209H322N56O67S6	4880.18	814.3705	6	25.0	2.55E+04	٧
M19	GIVEQC[*1]C[*2]TSIC[*1]S/FVNQHLC[*2]GSHLVEALYL	C137H214N36O43S4	3179.46	795.8729	4	25.0	1.76E+05	٧
M20	GIVEQC[*1]C[*2]TSIC[*1]SLY/HLC[*2]GSHLVEALY	C123H191N31O39S4	2854.28	952.4339	3	25.1	2.58E+04	
M21	GIVEQC[*1]C[*2]TSIC[*1]SLY/FVNQHLC[*2]GSHLVEALY	C146H223N37O45S4	3342.53	836.6388	4	25.4	1.07E+07	٧
M22	GIVEQC[*1]C[*2]TSIC[*1]SLY/LC[*2]GSHLVEALY	C117H184N28O38S4	2717.23	906.7501	3	25.6	2.72E+04	٧
M23	GIVEQC[*1]C[*2]TSIC[*1]SLY/FVNQHLC[*2]GSHLVEALY	C146H223N37O45S4	3342.52	836.6369	4	26.0	3.79E+04	٧
M24	GIVEQC[*1]C[*2]TSIC[*1]SLYQLENYC[*3]N/FVNQHLC[*2]GSHLVEALYLVC[*3]GER	C209H320N56O66S6	4862.17	973.4418	5	26.2	7.62E+04	
M25	CSLYQLENYC[*3]N / GSHLVEALYLVC[*3]GERGFFY	C158H228N38O47S3	3505.58	1169.5354	3	26.2	4.44E+04	
M26	GIVEQC[*1]C[*2]TSIC[*1]SLY/FVNQHLC[*2]GSHLVEALY	C146H223N37O45S4	3342.52	836.6381	4	27.0	1.82E+05	
M27	GIVEQC[*1]C[*2]TSIC[*1]SLY/FVNQHLC[*2]GSHLVEALYL	C152H234N38O46S4	3455.60	864.9078	4	27.4	1.18E+05	
M28	GIVEQC[*1]C[*2]TSIC[*1]SLYQLENYC[*3]N/FVNQHLC[*2]GSHLVEALYLVC[*3]GERGFF	C229H341N59O69S6	5213.33	1043.6723	5	28.0	2.32E+05	
M29	GIVEQC[*1]C[*2]TSIC[*1]SLYQLENYC[*3]N/ FVNQHLC[*2]GSHLVEALYLVC[*3]GERGFFY	C238H350N60O71S6	5376.40	1076.2866	5	28.0	2.75E+05	
	Derent [MIEL]E		E002 C2	1161 7220	E	27.2		-1

Insulin: Sites of Enzymatic Cleavage for Trypsin & Chymotrypsin Trypsin : C-Term Cleavage at K,R; Chymotrypsin: C-Terminal Cleavage at L,F,Y,W

Major digest products at 60 minutes

- M21 : GIVEQC[*1]C[*2]TSIC[*1]SLY / FVNQHLC[*2]GSHLVEALY (21% total)
- M4: QLENYC[*3]N / LVC[*3]GER (13%)
- M10: GFFYTPK (~2%)
- M11: GFFYTPKT (1.5%)
- Parent (1.2%)

Conclusions

• Detection and structural characterization of CP metabolites in biological matrix represent great analytical

• Recently released MetabolitePilot[™] 2.0 software employs multiple search mechanisms and automated

• Results from detection and identification of insulin metabolites formed in incubation with Trypsin and Chymotrypsin demonstrate that the software is capable of rapidly identifying metabolites of cyclic peptides • the application MetabolitePilot[™] software in identifying in-vivo metabolites of CP will be studied further

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