Comparative Proteome Analysis of Celastrol-Treated *Helicobacter pylori*

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Various preclinical and clinical trials have been conducted the efficacy of celastrol. In data presented in the current manuscript is the first trial to inhibit *Helicobacter pylori* with celastrol. In this study, the quantitative change of various *H. pylori* proteins including CagA and VacA by the anti-bacterial effect of celastrol was determined. The anti-*H. pylori* effects of celastrol was investigated by performing 2-dimensional electrophoresis and additional supporting experiments. After 2-dimensional electrophoresis analysis, spot intensities were analyzed and then each spot was identified using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) or peptide sequencing using Finnigan LCQ ion trap mass spectrometer (LC-MS/MS). The results show that celastrol has multiple effects on protein expression in *H. pylori*.

Key Words: *Helicobacter pylori*, 2 dimensional electrophoresis, Celastrol, MALDI-TOF-MS

Recently, interest in celastrol has renewed again as a new candidate drug for obesity and various chronic diseases (Cascao et al., 2017). Celastrol has been reported primarily to exhibit inhibitory effects on several cancers, such as breast cancer, prostate cancer and colorectal cancer (Shrivastava et al., 2015; Guo et al., 2015; Lin et al., 2016). And other preclinical and clinical trials on various purpose with celastrol have been continued till now (Fig. 1). This is the first trial to inhibit *Helicobacter pylori* with celastrol. Here we demonstrated the preliminary clues for showing anti-*H. pylori* effects of celastrol by performing 2-dimensional electrophoresis and some supporting experiments. To suggest celastrol as a potential cure drug for *H. pylori* infection, we tried to find the relationship between the quantitative expression level changes of human gastrointestinal disease-responsible proteins. In our data, the expression levels of some virulence factors were inhibited, while some other factors which were related to bacterial cell survival were increased as

'compensatory hyperincrease'.

In *H. pylori*, cytotoxin-associated protein A (CagA) and vacuolating cytotoxic protein A (VacA) have been reported as the most representative virulence factors because they are responsible for gastric or duodenal cancer development directly (Censini et al., 1996; Cover et al., 1993). While, in our results of 2-dimensional electrophoresis (2-DE), metabolic proteins like as AimE, FtnA, AcnB, type III restriction enzyme R protein, HSP90, UreB, OorA, including toxic protein CagA and VacA were showed quantitative changes when celastrol were treated to *H. pylori* culture. In this study, 2-dimensional electrophoresis (2-DE) and protein identification technique was used to analyze the effect of celastrol on the expression of various *H. pylori* proteins.

In this study, celastrol (Extrasynthese; Lyon, France) was dissolved in sterile dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis, MO, USA) as a 100 mM stock solution and stored at -80°C prior to use. And *Helicobacter pylori*

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reference strain 60190 was purchased from American Type Cell Collection (ATCC; Manassas, VA, USA). Bacteria were maintained under microaerophilic conditions at 37°C on Brucella agar plates (Becton-Dickinson, Braintree, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco,

Long Island, NY, USA). For experiments, *H. pylori* were cultured in Mueller-Hinton broth (Becton-Dickinson) with (3 µM, under the minimum inhibitory concentration) or without celastrol containing 10% FBS under microaerophilic condition for 3~4 days at 37°C and 100% humidity (Fig. 2).

To perform the proteomics study, the harvested samples were suspended in 0.5 ml of 50 mM Tris buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 16 µl protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IA, USA). For 2-DE analysis, pH 4~7 IPG strips (Amersham, Pittsburgh, PA, USA) were used. The 2-DE separation was performed on 8~16% (v/v) linear gradient SDS-polyacrylamide gels. Protein spot detection and 2DE pattern matching were carried out using ImageMaster™ 2DE Platinum software (Amersham). For comparison of protein spot densities between control and treated samples, more than 20 spots throughout all gels were correspondingly landmarked and normalized. The quantified spots of candidate proteins were compared with the aid of histograms.

Fig. 1. Chemical structure of celastrol.

Fig. 2. Minimum inhibitory concentration of celastrol to *H. pylori*.

For ensuring the reproducibility of 2-DE experiments, each sample was analyzed in triplicate. The procedure for in-gel digestion of protein spots from Coomassie Blue stained gels was carried out and these procedures were performed by ProteomeTech (Seoul, South Korea). In brief, protein spots were excised from stained gels and cut into pieces. The excised protein spots from a silver stained gel were cut into pieces and in-gel digested with trypsin.

To analyze the peptides, mass measurement of tryptic peptides was carried out with a Voyager-DE STR mass spectrometer (PerSpective Biosystems, Bedford, MA, USA). Close external calibration was performed for every samples with calibration mixtures. The proteins were identified by peptide mass fingerprinting (PMF) searching, against the Swiss-Prot and NCBI databases, using the search program ProFound (http://129.85.19.192/profound_bin/WebProFound.exe, Rockefeller University, Version 4.10.5), MASCOT (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF), or MS-Fit (<http://prospector.ucsf.edu/ucshtml4.0/msfit.htm>, University of California San Francisco, Version 4.0.5).

To identify the results, tryptic peptides were separated and analyzed using reversed phase capillary HPLC directly coupled to a Finnigan LCQ ion trap mass spectrometer (LC-MS/MS). For tandem mass spectrometry, a full mass scan range mode was $m/z = 450 \sim 2,000$ Da. The individual spectra from MS/MS were processed using the TurboSEQUENT software (Thermo Quest, San Jose, CA). The generated peak list files were used to query either MSDB database or NCBI using the MASCOT program (<http://www.matrixscience.com>). Only significant hits as defined by MASCOT probability analysis were considered initially.

Numerous protein spots (approximately 200) per silver-stained gel were analyzed by the PDQUEST program. Then

Table 1. Representatively increased proteins of *H. pylori* 60190 strain in the structure-bound fraction when *H. pylori* was exposed to celastrol

Spot No.	Protein ^{a)}		2-DE data ^{b)}			
	TIGR locus name	Protein name	pI / M _r	Protein-change ratio (%) ^{c)} after C3G treatment	Standard ^{d)} deviation	Reference
1	HP0786	Preprotein translocase subunit SecA (SecA)	6.12 / 99,207	- 146.90	1.73	Kim et al., 2015
2	HP0072	Urease subunit beta (UreB)	6.09 / 61,645	- 2099.90	3.23	Backert et al., 2005
3	HP0809	Flagellar basal body protein FlhL (FlhL)	5.41 / 55,134	877.53	1.74	Kim et al., 2015
4	HP0210	Heat shock protein 90 (Hsp90)	5.73 / 71,230	- 4863.70	4.93	Kim et al., 2015
5	HP0779	Aconitate hydratase AcnB (AcnB)	6.44 / 118,230	- 4237.40	13.49	Backert et al., 2015
6	HP0294	Acylamide amidohydrolase (AmiE)	7.28 / 37,689	- 434.36	16.04	This study
7	HP0589	2-oxoglutarate-acceptor oxidoreductase subunit (OorA)	6.94 / 41,448	- 4503.70	9.87	This study
8	HP0389	Iron-dependent superoxide dismutase (SodB)	6.17 / 24,617	- 8120.30	13.68	Park et al., 2016
9	HP0653	Non-heme iron-containing ferritin (FtnA)	4.86 / 19,302	- 179.13	37.32	This study

a) Protein designation according to *H. pylori* strain, NCBI (<http://www.ncbi.nlm.nih.gov/>), KEGG database (<http://www.genome.jp/kegg/>).

b) According to peptide mass fingerprinting (PMF) data were obtained from Figure 1.

c) The ratio of protein-change amount was calculated as follows:

$$\text{change ratio (\%)} = \frac{\Delta \text{ value (protein amount w/ celastrol - protein amount w/o celastrol)}}{\Delta \text{ value (protein amount w/o C3G)}} \times 100$$

d) Standard deviation of protein-change ratio in each spot was obtained from repeated five independent 2-DE analysis.

Table 2. Representatively decreased proteins of *H. pylori* 60190 strain in the structure-bound fraction when *H. pylori* was exposed to celastrol

Spot No.	Protein ^{a)}		2-DE data ^{b)}			
	TIGR locus name	Protein name	pI / M _r	Protein-change ratio (%) ^{c)} after C3G treatment	Standard ^{d)} deviation	Reference
1	HP0547	Cytotoxin-associated gene protein A (CagA) fragment	5.75 / 81,500	+ 4784.72	9.98	González et al., 2013
2	HP0887	Vacuolating cytotoxic protein A (VacA) fragment	6.65 / 87,970	+ 2305.02	3.84	Backert et al., 2005
3	HP0072	Urease subunit beta (UreB)	6.09 / 61,645	+ 1546.73	2.48	Kim et al., 2015
4	HP0294	Acylamide amidohydrolase (AmiE)	7.28 / 37,689	+ 653.95	69.09	This study
5	HP0589	2-oxoglutarate-acceptor oxidoreductase subunit (OorA)	6.94 / 41,448	+ 445.27	23.22	This study
6	HP0010	Heat-shock protein 60 subunit (GroEL))	6.17 / 20,188	+ 2492.67	1.44	Park et al., 2016

a) Protein designation according to *H. pylori* strain, NCBI (<http://www.ncbi.nlm.nih.gov/>), KEGG database (<http://www.genome.jp/kegg/>).

b) According to peptide mass fingerprinting (PMF) data were obtained from Figure 1.

c) The ratio of protein-change amount was calculated as follows:

$$\text{change ratio (\%)} = \frac{\Delta \text{ value (protein amount w/ celastrol - protein amount w/o celastrol)}}{\Delta \text{ value (protein amount w/o C3G)}} \times 100$$

d) Standard deviation of protein-change ratio in each spot was obtained from repeated five independent 2-DE analysis.

methodologically the selected each protein spot was relatively evident than others in the 2-DE images and were identified by peptide mass fingerprinting using MALDI-TOF-MS or by peptide sequencing using LC-MS/MS (Table 1, 2). All the analyzed 2-DE spot profiles of *H. pylori* 60190 strain among total spot profiles of protein changes were compared to reference data (Choi et al., 2002; Jungblut et al., 2000). The displayed protein spots in this study were focused on the relation with clinical diseases, such as chronic gastritis, gastric ulcers, or gastric adenocarcinoma (Park et al., 2006; Backert et al., 2005).

We could find meaningful nine protein spots which the expression level was decreased by celastrol treatment. They were preprotein translocase subunit SecA (SecA), Urease subunit beta (UreB), flagellar basal body protein FliL (FliL), heat shock protein 90 (Hsp90), aconitate hydratase AcnB (AcnB), acylamide amidohydrolase (AmiE), 2-oxoglutarate-acceptor oxidoreductase subunit (OorA), iron-dependent superoxide dismutase (SodB), and non-heme iron-containing ferritin (FtnA) (Fig. 3 and 4).

SecA; Sec-related proteins are associated with the type V secretion system (T5SS) of *H. pylori* (Cover and Blanke,

2005). It has been reported that VacA secretion is SecA-dependent (Cover and Blanke, 2005; Shaffer et al., 2011). This suggests that the decreased SecA expression might affect the accumulation of VacA in cell. We could also find such a phenomenon in our data (Fig. 3 and 4).

UreB; Urease subunit beta with subunit alpha involve in critical role of *H. pylori* colonizing in acidic gastric environment (Park et al., 2006). It has been reported that *H. pylori* ureases produces alkaline ammonia by degrading urea. *H. pylori* can survive in acidic gastric environment thereby neutralizing acidic gastric environment with this alkaline substance. If the expression level of UreB was decreased by celastrol treatment, it showed that celastrol might be a potential inhibitor for *H. pylori* (Fig. 3 and 4).

FliL; although the function of FliL in *H. pylori* is not clear yet, it has been reported that FliL is essential for swarming but not for swimming or motility in genus *Proteus* (Lee et al., 2013). It is thought that *H. pylori* FliL is also a trans-membrane protein located adjacent to the basal body of its flagellar structure (Lee and Belas, 2015). When *H. pylori* was exposed to celastrol if the expression of FliL was decreased, it might be an expected positive result (Fig. 3 and 4).

Fig. 3. A representative two-dimensional gel-electrophoresis map of the whole-cell protein (30 μ g) of *H. pylori* 60190 standard strain isolate. This showed the changed expression levels of various proteins when *H. pylori* was exposed to 3 μ M of celastrol for three days. The spots were identified by MALDI-TOF-MS or TOF-MS. Molecular size markers are shown on the left in kDa. (A) Analysis of quantitatively decreased protein spots. (B) Analysis of quantitatively increased protein spots.

Hsp90; *H. pylori* Hsp90 showed decrease in its expression with celastrol in our data (Fig. 3 and 4), although its function in *H. pylori* is not known yet.

AcnB, AmiE, OorA, SodB and FtnA; these molecules are important metabolic components in *H. pylori*. Thus, it was thought that the growth of *H. pylori* might be suppressed thereby inducing metabolic malfunction in *H. pylori* by celastrol treatment (Fig. 3 and 4).

Some other six proteins which are related to cell survival

or pathogenicity were showed increased as irony. Because the celastrol was treated to *H. pylori* culture under the minimum inhibitory concentration (3 μ M), it was thought that bacterial cells might fight for survival when they were exposed to celastrol. Here we explained this as 'compensatory hyper-increase'.

Cytotoxin-associated gene protein A (CagA), Vacuolating cytotoxic protein A (VacA), UreB, AmiE, OorA, and Heat-shock protein 60 subunit (GroEL) were showed increased

Fig. 4. Quantitatively changed protein spots and protein identification. (A) SecA, UreB, FliL, Hsp90, AcnB, AmiE, OorA, SodB, FtnA were quantitatively decreased when celastrol was treated to *H. pylori* 60190. (B) CagA, VacA were accumulated in periplasmic or in cytoplasmic space of *H. pylori* 60190 when celastrol was treated. And OorA, AmiE, UreB, GroEL were also increased when celastrol was treated to same conditions. It was thought as 'compensatory hyperincrease' to recover the chemical stress.

protein expression level when celastrol was treated. It was thought that CagA, VacA were accumulated in periplasmic or in cytoplasmic space of *H. pylori* 60190.

CagA and VacA; they toxin-like proteins of *H. pylori* are strongly responsible for significantly increased risk of the development of gastric disorders like chronic gastric inflammation, ulcers, various cancers (Censini et al., 1996). Although CagA accumulation in cell could not be explained in the aspect of its mechanism, here, in our data, VacA accumulation in cell could be explained readily. Because VacA secretion is regulated by SecA-dependent T5_aSS, as shown prior, if SecA was decreased by exposing celastrol, its accumulation in cell would be induced (Kim et al., 2014) (Fig. 3 and 4).

Now, the complete genome sequences of *H. pylori* strains 26695 and J99 had been reported (Alm et al., 1999; Tomb et al., 1997), however the information is imperfect. Thus it is thought that the additional genomic or proteome information like this should be accumulated for further research.

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CONFLICT OF INTEREST

No conflicts of interest, financial or otherwise, are declared by the authors.

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