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Instructions for use

A novel sponge-derived protein thrombocorticin is a new agonist for thrombopoietin receptor

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ABSTRACT

We screened 868 marine extracts in search of hematopoietic molecules resulted in findings of several extracts that proliferated Ba/F3-HuMpl cells but not the cells expressed with other hematopoietic cytokine receptors, EPO and G-CSF. Separation of the most potent extract of a Micronesian sponge *Corticium* sp., guided by the cell proliferation assay using Ba/F3-HuMpl cells resulted in an isolation of thrombocorticin (ThC), a novel 14 kDa protein as an active principal. ThC displayed concentration-dependent proliferation of Ba/F3-HuMpl cells, and had a stronger activity than that of eltrombopag, a small molecule drug used to treat thrombocytopenia. ThC induced phosphorylation of STAT5, suggesting that it activates Jak/STAT pathway as in the case of TPO. These results together indicated that ThC is a specific agonist for c-Mpl, although the size and shape differs largely from TPO. Here we present isolation, characterization and biological activity of ThC.

1. Introduction

Mammalian blood is composed largely of red and white blood cells, as well as platelet and lymphoid cells. Although these cells have discrete functions, they all are derived from hematopoietic stem cells (Orkin, 2000). Upon exposure to various hematopoietic cytokines including erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), and thrombopoietin (TPO), the cells differentiate and maturated into red, white blood cells and to platelets, respectively. Dysfunction in hematopoiesis causes various disorders including renal anemia, neutropenia, or thrombocytopenia. Therefore, molecules that regulate hematopoietic signal transductions are of great interest as to both biomedical and therapeutic points of view. Several protein-based and small molecular drugs are clinically available to treat hematopoietic disorders. Protein drugs that based recombinant human EPO (Coleman and Brines, 2004) and a modified recombinant human G-CSF (Amadori et al., 2005) are widely used to treat anemia and neutropenia, respectively. Contrary to the above successful examples, use of recombinant human TPO was limited in clinic due to rapid development of antibody that cross-react with endogenous TPO (Li et al., 2001). Besides cytokines, small synthetic peptides with 14-amino acid residues were, surprisingly, found to behave as agonists for EPO receptors (Wrighton et al., 1996). Shortly after, other homologous peptides were also found to serve as TPO receptor agonist (Cwirla et al., 1997). Although the peptides themselves have failed to become clinically useful due to their short half-life *in vivo*, a TPO receptor agonist 14-mar peptide was eventually developed as an immunoglobulin-conjugated form called "peptibody" that largely improved the half-life issue (Stasi et al., 2008). Romiplostim, the peptibody drug has approved and it is widely used despite its proteinous nature (Molineux, 2011).

Non-peptidyl small molecules that may allow oral administration and favorable pharmacodynamics are advantageous as drug, and thus extensive searches for such compounds with agonist activity for hematopoietic cytokine receptors have been sought (Sakai et al., 2006). Interestingly, a number of small molecules were found to be good agonists for TPO receptor (c-Mpl (Wendling et al., 1994)) whereas only a few compounds were found to activate EPO (Qureshi et al., 1999) or G-CSF (Tian et al., 1998) receptors. Among the small TPO receptor agonists, a compound found from dyestuff library was extensively revolved to develop eltrombopag (Duffy et al., 2001; Duffy and Erickson-Miller, 2007), the first clinically used c-Mpl agonist drug now widely used to treat thrombocytopenia such as idiopathic thrombocytopenic purpura (ITP) (Bussel et al., 2007).

These small molecular drugs were all found from library owned by pharmaceutical companies and are mostly synthetic compounds (Sakai et al., 2006). We thus searched for potential hematopoietic agents from natural sources because natural products often provide unexpected structural features that lead to discovery of novel pharmacophores. For example, a screening of extracts from fungus associated with marine benthic organisms resulted in an isolation of xanthocillins, well known structure but a novel pharmacophore, from marine-derived fungus *Basipetospora* sp. as c-Mpl agonist (Sakai et al., 2005). This finding prompted us to search further for potential hematopoietic compounds from natural sources. We thus extended the screening study using our marine extract library and found that aqueous extracts of marine sponge showed potent TPO-like activity. Here we describe isolation and physico-chemical characterization of thrombocorticin (ThC), a new protein that can activate thrombopoietin receptor.

2. Results

2.1. Screening of extracts from marine benthic organisms.

We tested cell proliferation activity of our marine extract library to search for compounds with hematopoietic activities using Ba/F3 murine pro-B lymphoma cell lines. Ba/F3 cells natively express interleukin-3 (IL-3) receptor and proliferate only in the presence of the cytokine. Transfection of gene encoded each human EPO receptor (EPO-R), c-Mpl (TPO-R) or G-CSF-R, results in expression of hEPO-R, hTPO-R and hG-CSF-R on the cell surface, and the cell proliferates in response to IL-3, EPO, TPO or G-CSF, respectively. These cell lines, therefore, are proven to be useful to evaluate molecules that interact with hematopoietic receptors. The cytotoxicity of the extract was first determined by using Ba/F3 cells in the presence of IL-3. Potential hematopoietic activity was evaluated at sub-toxic concentrations of the extract using cells expressed with each hEPO-R, c-Mpl and hG-CSF-R. These cells cannot proliferate and die within two to three days in the absence of appropriate hematopoietic cytokines. When the extract contains compound with the cytokine-like activity, cells are expected to proliferate even in the absence of cytokines. In the present study, we tested aqueous and methanol extracts of marine organisms collected in Republic of Palau and in Chuuk, or Yap State Micronesia, or in Kyusyu, Shikoku and Okinawan coasts in Japan (Table 1).

Table 1.

Number and classification of samples used in this study

	Classifications				total
	sponge	tunicate	coelenterate	other	
Palau	244	47	17	5	313
Chuuk	233	23	4	6	266
Yap	68	0	0	0	68
Japan	201	4	1	15	221
total	746	74	22	26	868

Among the tested, only a small number of extracts exhibited the activity: five Chuuk, two Palau and one from Japan exhibited potent proliferation activity selectively for c-Mpl cells (Table 2). It should be noted, however, that the active five Chuuk specimens are the same sponge identified as *Corticium* sp. We did not find any extract that proliferate Ba/F3-HuEPOR or Ba/F3-HuGCSFR cells, whereas each one extract from Chuuk (sponge) and Kyushu (tunicate) proliferated all three Ba/F3 cell lines tested (Table 2). Interestingly extracts from other specimens of *Corticium* sp. collected in Palau and Yap did not exhibit any proliferation activity.

In summary, we found a total of four extracts of sponge species that selectively proliferated Ba/F3-HuMpl cells. One sponge and one tunicate extracts activated all the Ba/F3 derived cell lines. Two Palauan sponges had activity in both methanol and aqueous extracts, but other active extracts were all aqueous.

Table 2.

Number of species activated each of the three types of Ba/F3 cells

	c-Mpl (TPO)	EPO	G-CSF
Palau	2	0	0
Chuuk	2 (1)	1(1)	1(1)
Yap	0	0	0
Japan	2 (1)	1(1)	1(1)

Number of sample that proliferate all three cells are in parentheses

2.2. Isolation of Thrombocorticin ThC

Because the extracts from *Corticium* sponges collected in Chuuk were the most potent among the hits, we chose to work on those extracts further. The sponge specimen was extracted with water to give black extract presumably due to melanin in the sponge. The extract was dialyzed against water, to give small- and macromolecular fractions. Because we confirmed that the macromolecular fraction of the sponge stimulated the proliferation of Ba/F3-HuMpl cells, but not Ba/F3 itself, Ba/F3-EPO, nor Ba/F3-G-CSF cells (supplementary data Fig. S1), further separation was guided by Ba/F3-HuMpl assay. Because this result suggested a proteinous nature of the active principal, we next assessed the chromatographic behavior of the active principal using either Sephadex LH-20 or DE52 anion exchange column, then the SDS-PAGE of representing fractions were compared. In both separation modes, active fraction contained bands around 14 kDa (Fig. 1). Note that columns were stained in black in both modes. Rather surprisingly, Sephadex LH-20 successfully separated active proteins.
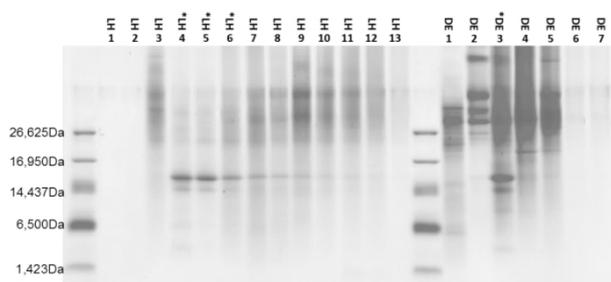

Fig. 1. Protein profiles monitored on a SDS-PAGE for fractions obtained from Sephadex-LH20 (LH1-13) and DE-52 anion exchange column (DE1-7). Fraction with the cell proliferation activity was indicated *.
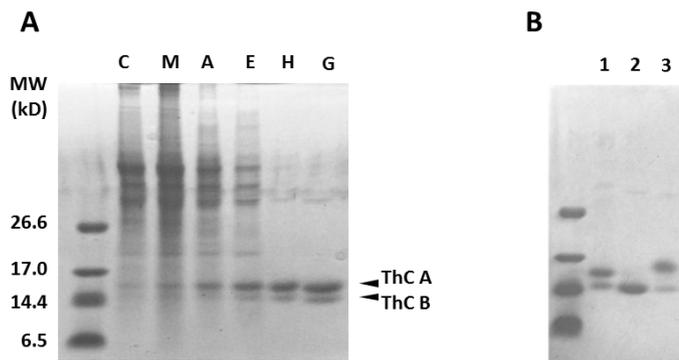
Fig. 2. A. SDS-PAGE for crude extract (C) of the sponge, the macromolecular fraction after dialysis (M), supernatant after acid treatment (A), that after anion exchange chromatography (E), and hydrophobic chromatography (H), and final purification by gel filtration (G). **B**. SDS-PAGE for purified ThC: with mercaptoethanol and heat treatment (lane 1), with mercaptoethanol but without heat treatment (lane 2), without mercaptoethanol and heat treatment (lane 3).

We further optimized the separation scheme to prevent permanent staining of the columns by melanin, and to be able to handle larger amount of the extract. In this process, we found that the active proteins can be readily separated from other proteins by precipitating by acidic buffer. Thus, the macromolecular fraction from dialysis was suspended in glycine buffer (pH 3.0) to precipitate out other proteinous components. Since the activity was safely recovered exclusively in the supernatant which contained the 14 kDa bands, it was separated on an anion exchange chromatography using DE52 followed by hydrophobic column to afford active fraction. Final separation by a gel filtration column afforded the active protein named thrombocorticin (ThC). ThC could also be purified by using anion exchange column, in that the fraction containing ThC from the Sephadex-LH20 separation was purified by a Mono-Q column to give active protein. SDS-PAGE of ThC exhibited two bands at around 14 kDa in the presence of mercaptoethanol, thus each was denoted as ThC-A and ThC-B, respectively (Fig. 2). Interestingly, however, only ThC-B was observed under non-reduced condition, while ThC-A was observed in the same condition without heating (Fig. 2). These results suggested that reduced ThC can exist as two conformationally different forms.

2.3. Physico-chemical characterization of ThC

MALDI-TOFMS for ThC had broad ion clusters at m/z = 13,669 [M+H]$^+$ as well as those for [2M+H]$^+$, [3M+H]$^+$, [4M+H]$^+$ (Fig. 3A). We have observed similar ladder-like ion clusters previously in the MALDI-TOFMS experiment of CchG, a marine sponge-derived galectin (Ueda et al., 2013), indicating these proteins form non-covalent complex in the MALDI condition. An ESI-Orbitrap MS showed ions for [M + 6H]$^{6+}$ to [M + 9H]$^{9+}$, being the [M + 8H]$^{8+}$ as a major ion species. A deconvoluted spectrum showed the presence of major ion with molecular weight of m/z = 13,665 but several minor ions were also observed indicating that the active protein we have obtained contains several minor orthologues (Fig. 3B).
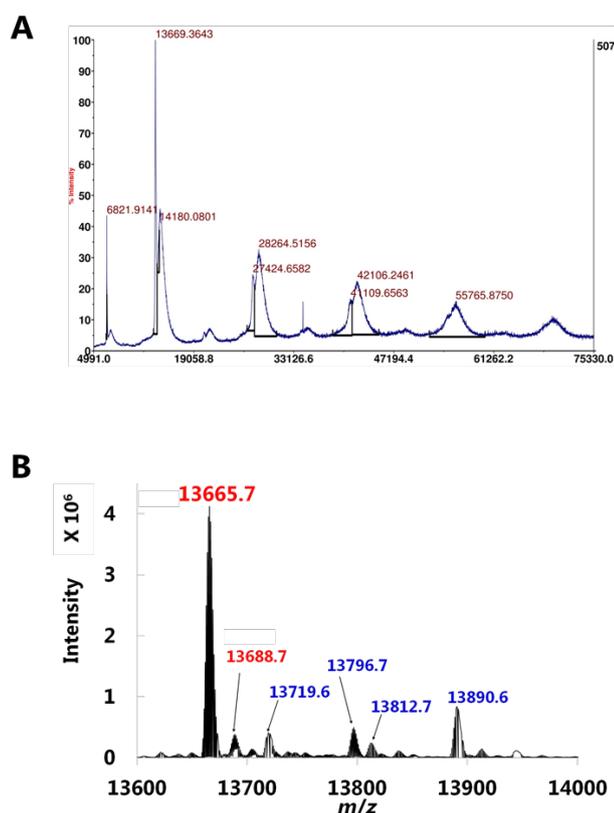
Figure 3. Mass spectral data for the purified protein **A.** MALDI-TOF and **B.** A deconvoluted ESI-Orbitrap mass spectrum showing molecular ions ([M+H]$^+$ and [M+Na]$^+$) for ThC (red) and putative orthologues (blue).

We therefore used this sample for further experiments, since purification of this sample using HPLC could not remove small amount of the orthologous proteins. Molecular size of ThC was estimated by the gel filtration experiment to be about 25 kDa, suggesting that the native ThC forms a dimeric complex (Fig. S2). Treatment of ThC with dithiothreitol (DTT) followed by iodoacetamide (IAA) resulted in formation of alkylated derivative whose molecular ion shifted from m/z 13,667 of native protein to 13,773 by ~106 Da (Fig. S3), suggesting that one intramolecular disulfide bond presents in the protein.

We next determined *N*-terminal amino acid sequences of ThC. Because SDS-PAGE had a major band ThC-A, and minor band ThC-B, each of the band was cut and subjected to automated Edman degradation to give a tentative sequences: TAXTTGPQQIIPPAA for ThC-A and TAXTTGPXTIIS for ThC-B. These results suggested that ThC-A and B were possibly the same protein, as suggested by the behaviors in SDS-PAGE or orthologous proteins, however, low signal intensity in some amino acids in the sequence analysis hampered rigorous determination. We thus attempted to obtain cDNA using a preserved sample in RNAlater to amplify gene responsible for the production of ThC in the sponge by PCR. Unfortunately, however, construction of cDNA library was not successful presumably due to presence of a large amount of melanin in this species that interfered with mRNA extraction and PCR experiments, and thus improved RNA extraction methods should be applied for future investigations (Islas-Flores et al., 2006). The *N*-terminal amino acid sequences did not have any similarity with previously known proteins, suggesting that ThC is a new compound.

2.4. Biological activity of ThC.

2.4.1. Proliferation of Ba/F3-HuMpl cells by ThC

ThC prompted proliferation of Ba/F3-HuMpl cells in a concentration dependent manner. EC_{50} values determined from datasets obtained at pH 7.0, 8.5 and 10 were 22.8, 10.8, and 10.8 nM, respectively (Fig. 4). No proliferation activities for Ba/F3-HuEPOR, Ba/F3-HuGCSFR cells or Ba/F3 cells were observed (data not shown). The cell proliferation activity decreased upon heat treatment of the protein, in that the activity was lost gradually by treatment of the protein at 98°C for 10, 30, and 60 min (Table 3). ThC was stable under the acidic condition since acid treatment at pH 3.0 was safely used during the separation. These data indicated that ThC is a small and stable protein. Because ThC behaved similarly to CchG a sponge derived galectin in the MALDI-TOF MS analysis, we tested hemagglutinating activity of the protein. ThC, however, did not aggregate rabbit erythrocyte even at the highest concentration tested (0.5 mg/mL) indicating that the protein has no lectin-like activity. The stability observed here suggests some structural features of ThC including increases in number of hydrogen bonds, ion pairs, and other factors that contribute to protein stability (Tanaka et al. 2006).
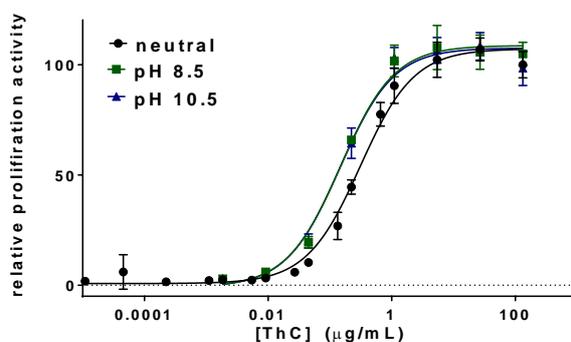

Fig. 4. Concentration-dependent proliferation activity of ThC for Ba/F3-HuMpl cells. Activity was shown relative to TPO at 10 ng/mL (100 %). Each data point is triplicate. Bar ± SD.

Table 3.

EC_{50} values for ThC under various conditions

	EC_{50} (CI)[a]	EC_{50}[b]
pH 7.0	0.31 (0.26 ~ 0.37)	22.8
pH 8.5	0.15 (0.11 ~ 0.20)	10.8
pH 10	0.15 (0.10 ~ 0.20)	10.8
98 °C, 10 min	0.49 (0.39 ~ 0.61)	35.9
98 °C, 30 min	1.78 (1.53 ~ 2.09)	130
98 °C, 60 min	7.65 (6.58 ~ 8.92)	562

[a] μg/mL, (CI, 95 % confident interval). [b] nM

2.4.2. Signal transduction by ThC

Because selective proliferation of Ba/F3-HuMpl cells by ThC suggested that the protein act as agonist for TPO-R. Thus the phosphorylation of STAT (Signal Transducers and Activator of Transcription) 5 was assessed by Western blotting analysis, since activation of cMpl results in phosphorylation of Jak (Janus kinase)-2 which in turn phosphorylates STAT5. A dimer of phosphorylated STAT5 finally functions as a transcriptional factor giving rise to cell proliferation. Ba/F3-HuMpl cell was treated with ThC, and the cell lysates were analyzed by Western blotting with antibody against phosphorylated STAT5 (p-STAT5). ThC induced phosphorylation of STAT5 indicating that it stimulated TPO receptor protein c-Mpl on the cell surface to transduce signals via Jak/STAT signaling pathway.
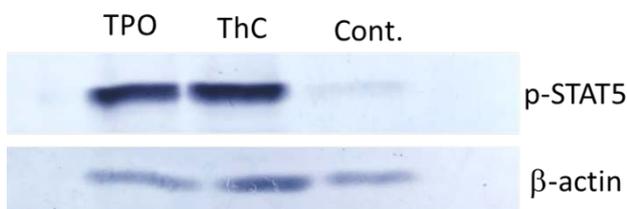

Fig. 6. STAT5 activity by ThC and TPO in Ba/F3-HuMpl cells. Control (Cont.) is untreated cell lysate.

3. Discussion

A screening of methanol and aqueous extracts from a total of 868 marine benthic organisms for cell proliferation activity of Ba/F3-HuEPOR, Ba/F3-HuGCSFR, Ba/F3-HuMpl, models of hematopoietic stem cells, resulted in four marine species with selective proliferation activity against Ba/F3-HuMpl, two species with proliferation activity for all the cells tested. No extract with proliferation activity selective for Ba/F3-HuEPOR, Ba/F3-HuGCSFR was found. Two species of Palauan sponges showed activity both in methanol and aqueous extracts. The aqueous extracts of one sponge from Chuuk and one tunicate from the Kyushu proliferated all the Ba/F3-derived cells, including Ba/F3 cell itself. Active compounds in these extracts can either be non-selective agonist that could stimulate all the hematopoietic receptors or an agonist for IL-3 whose receptor was naturally expressed on the Ba/F3 cells. Identification of active compounds from these species is now under investigation in our group and will be reported elsewhere. Results from the screening study illustrated the difficulty of finding molecules to interact with hematopoietic cytokine receptors; however, this outcome was not surprising to us as this class of receptors have been known to be difficult targets in drug discovery (Schreiber and Walter, 2010). Nevertheless, discovery of a few extracts that can stimulate Ba/F3-HuMpl cells are remarkable as no other natural products, except for xanthocillins, have been known to the best of our knowledge. Furthermore, our finding of novel protein, ThC, that activates TPO receptor is notable as no exogenous protein that possess cytokine-like activity have been known. It should be noted however, small 14-mer peptides were found to activate EPO and TPO receptors (Wrighton et al., 1996). These peptides are known to interact with ligand binding domain of TPO receptor and facilitate dimerization of the receptor to

form an activated complex. In fact, the dimers of these peptides are far more active than the monomers (Cwirla et al., 1997). These observations led to a development of clinical drug, romiplostim, a hybrid of the small peptide and immunoglobulin Fc fragments (Molineux, 2011).

TPO receptor can also be activated in the other way; eltrombopag, the first commercialized small molecular c-Mpl agonist interacts with a trans-membrane domain of TPO receptor (Drachman and Kaushansky, 1997). Few other small molecular drugs including NIP-022, YM477 and butyzamide are now under clinical trials, but their mechanism of actions are similar to that of eltrombopag (Ikeda and Miyakawa, 2009). In the present work we showed that ThC stimulates the cell proliferation through the Jak/STAT signaling by interacting specifically with cMpl, like other agonists. A comparison of the cell proliferation activity of ThC and other TPO mimetic compounds indicated that the activity of ThC is as potent as other drugs, suggesting that the specific binding of ThC to the receptor facilitates its effective receptor activation. Although detailed mechanism of action is yet to be determined, the high water solubility as well as proteinous nature of ThC preclude its binding to the transmembrane domain. The binding of ThC to the receptor binding domain is likely while no experimental evidences are available at this point. Further investigations including determination of complete amino acid sequence and three dimensional structure of ThC, as well as its mechanism of receptor activation are now in progress in our laboratory.

Table 4

A comparison of cell proliferation activity for TPO and TPO mimetic compounds

Compound	type	MW (Da)	EC$_{50}$ (nM)	Drug status
Romiplostim (Broudy and Lin, 2004)	protein	50 k	0.2 [a]	marketed
rhTPO	protein	18.6k	0.1[a]	-
ThC	protein	14k	10[a]	-
AF12505 (Cwirla et al., 1997)	peptide	1754	400[a]	
AKR501 (YM477) (Fukushima‑Shintani et al., 2009)	syn	649	3.3[a]	developing
Butyzamide (Nogami et al., 2007)	syn	591	10[a]	Approved (Kim, 2016)
NIP-004 (Nakamura et al., 2006)	syn	455	50[b]	-
Eltrombopag (Erickson‑Miller et al., 2009)	syn	442	270[a]	marketed
Xanothocillin (Sakai et al., 2005)	nat	302	13[b]	-

[a]tested on Ba/F3-HuMpl or [b]UT7/TPO, UT7/EPO-mpl cells. syn: synthetic molecule, nat: natural product.

4. Conclusion

In conclusion, ThC we found here from the marine sponge was shown to be a new agonist of c-Mpl. The potency of ThC *in vitro* contest other newly developed drugs and drug candidates and exceeds some of the approved drugs, showing its effective receptor activation. It is not known, however, whether ThC promotes differentiation of blood progenitor cells into megakaryocyte and lead to a formation of platelet, and whether it contributes to platelet activation. Discovery of plural marine extracts with TPO, and IL-3-like activity suggested that diverse of molecules can activate certain group of hematopoietic cytokine receptors. Further studies for mechanism of activation by ThC and other natural products would add deep insight into mode of activation of hematopoietic cytokine receptors.

5. Experimental

5.1. General Procedures

Tris-tricine SDS-PAGE was performed as described previously (Schagger and von Jagow, 1987).. The protein bands were stained 0.25% Coomassie Brilliant Blue. BioLogic Duo Flow system (BioRad, Hercules, CA, USA) equipped with QuadTec UV/Vis detector was used for protein separation. An HPLC system equipped with photodiode array detector (Shimazu Kyoto, Japan) was used for separation/purification of protein or peptides. NanoDrop 1000 (Thermo Scientific, Waltham MA, USA). Protein concentration was estimated by assuming $A_{280} = 1$ corresponds to 1 mg/mL protein. For low resolution mass spectral analysis and peptide mass mapping, Matrix Assisted Laser Desorption/Ionization (MALDI) technique was employed using 4700 Proteomics analyzer (Applied Biosystems, Foster City, CA, USA) in a time-of-flight (TOF) linear high/MS mode. α-Cyano-4-hydroxycinnamic acid (CHCA) was used as a matrix. High resolution ESI-MS spectrum was measured on LTQ-Orbitrap XL (Thermo Scientific).

5.2. Biological materials.

Sponge specimens were collected by SCUBA in Chuuk and Yap state Micronesia, Republic of Palau, Iriomote Okinawa and various locations in Kyushu and Shikoku areas in Japan. Samples were stored in freezer (-20 °C) until use. All collections were made under permission of local governments. The specimen that showed potent activity was identified as *Corticium* sp. Immediately after the collection of the sponge (CHK151) a portion of specimen was cut in piece (1 mm) and several pieces were stored in RNAlater (Quiagen, Venlo, Holland) for cDNA preparation.

5.3. Extraction and isolation

The sponge specimen (CHK129, 53.3 g) was lyophilized and the dried sample (11 g) was extracted with water to give an aqueous extract (4.4 g). This material was dialyzed against water using cellulose tube (UC36-32-100, 50 Å pore) to give an inner (1.8 g, CHK129-inner) and an outer fraction (3.0 g). Similarly other sample (CHK151, 10.8 g) was extracted and processed to give inner fraction (320 mg, CHK151-inner). To assess chromatographic behavior of bioactive compounds, the later extract (59.2 mg) was first applied on a Sephadex LH20 column (2.5 x 114.5 cm) and eluted with water and

eventually combined into 13 fractions according to protein profiles on SDS-PAGE as well as bioactivity. The same extract (11 mg) was separated on an open column of DE52 (1.5 x 6.5 cm, GE healthcare Japan, Tokyo) using gradient of NaCl (0-1 M) in 0.1 M Tris-HCl, pH 8.0, 1 mL/min. Fractions were combined into seven sub-fractions.

For large scale separation, the inner portion of CHK129 (1.8 g) was suspended in an acidic buffer (200 mM Glycine-HCl buffer, pH 3.0) to precipitate most of the proteinous component. The supernatant was separated on an anion exchange chromatography using DE52 (2.5 × 24 cm, 0-0.4 M linear gradient of NaCl in 20 mM Tris-HCl, pH 8.0, 1 mL/min). The active fractions were combined, desalted and then lyophilized. The active fraction (7.5 mg) was separated by a gel filtration column TSKgel G2000SW (7.5 × 300 mm, TOSOH) eluting with PBS at flow rate of 1 mg/mL to give fraction ThC (3.1 mg).

5.4. Cell proliferation assay.

Murine Ba/F3 cell line and that transfected with human erythropoietin receptor gene, human G-CSF receptor gene, or human c-Mpl gene were established as previously described (Nakamura et al., 2006). Cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS and rmIL-3, rhEPO, rhTPO, or rhG-CSF. Cells were pre-cultured for 4 days with appropriate cytokine. The cells were harvested by centrifugation (1000 rpm, 3min) and rinsed with PBS, centrifuged, and then were suspended in the medium to 6×10^4 cells/mL. The cell suspension was transferred (100 µL) to 96-well microtiter plate and test sample 20 µL was added. For positive control rhTPO (Wako Pure Chemical, Japan, 60 ng/mL, 20 µL) was added and for negative control medium (20 µL) was added. The cells were cultured (37 °C, 5 % CO_2) for 4 days. Cell counting kit (Dojindo Japan, Kumamoto) was added (10 µL) to the wells and absorbance at 450 nm was measured using microplate reader. Relative cell proliferation was expressed as follows:

$$\text{Relative cell proliferation activity (\%)} =$$

$$(A450_{Sample} - A450_{Negative}) \times 100 / (A450_{Positive} - A450_{Negative})$$

Assay was performed in triplicate and mean ± SD was plotted to generate concentration-response curve using Prism software (GlaphPad, San Diego CA).

5.5. Hemagglutination assay

To a two-fold serial dilution of ThC in 96-well V-shape bottom microtiter plate, a suspension (4%, 20 µL) of rabbit erythrocytes in 20 mM Tris-HCl was added and the mixture was incubated at 37°C for 30 min. Final concentration of the highest ThC was set at 500 µg/mL.

5.6. Western blotting

The signal transducing activity of ThC through TPO receptor was assessed by SDS–PAGE and Western blotting. Ba/F3-HuMpl cells were washed 3 times with PBS and suspended in RPMI containing 10% FBS at a cell density of 1.5×10^6 cells/mL. The cell suspension was incubated in a CO_2 incubator (5% CO_2, 37 °C) for 12 h. To 3 mL of this cell suspension (5×10^6 cells), each TPO (final concentration, 100 ng/mL), ThC (final concentration, 50 μg/mL) and control buffer was added. After the mixture was incubated at 37 °C for 1 h, the cells were centrifuged, washed with PBS. The residual buffer was removed by centrifugation, and then the cell cooled in an ice bath was added the lysis buffer (500 μL, CST Japan, Tokyo). The cell lysate was sonicated and then centrifuged to collect the supernatant which was separated by SDS–PAGE (15%). The separated proteins were electro-blotted onto polyvinylidene difluoride (PVDF) membrane (0.2 mm pore size) at 15 V for 10 min and incubated with anti-p-STAT5 (CST Japan) followed by an alkaline phosphatase-labeled second antibody (CST Japan). The antigen–antibody complex formed on the PVDF membrane was visualized with BCIP-NBT Solution Kit for Alkaline Phosphatase Stain (Nacalai Tesque, Kyoto, Japan).

5.7. Estimation of molecular size

The above gel filtration column was used to estimate apparent molecular size of ThC. The standard protein mixture containing: triosephosphate isomerase (26,625); myoglobin (16,950); α-lactalbmin (14,437); (BioRad) was eluted using a phosphate buffer (50mM, pH 7.0 containing 0.15M NaCl, 1 mg/mL). The void volume of the column (V_0 = 5 mL), bed volume (V_t =10 mL) was used to determine Kav. Apparent molecular size was estimated from the calibration curve plotting Kav versus molecular size of the standards.

5.8. Amino acid sequence determination.

For the *N*-terminal sequence determination, a sample of ThC (159 μg) was separated on a tricine SDS-PAGE, and was blotted on a PVDF membrane, stained by Ponceau-S. The bands of ThC-A and B were each cut out from the membrane and analyzed by automated sequencer, Procise HT Protein Sequencing System (Applied Biosystems).

Appendix A. Supplementary data

Supplementary data to this article can be found online at 000

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