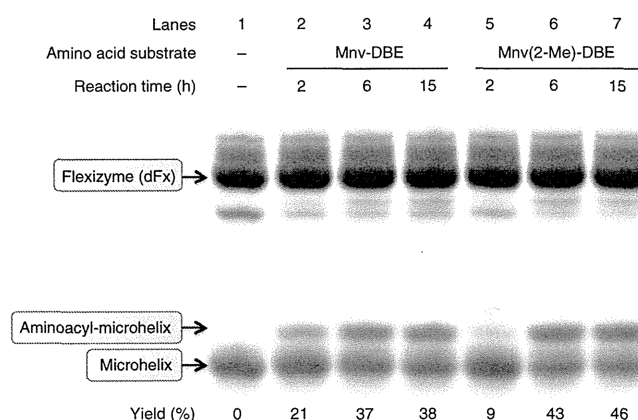

**Figure 3 | ** Acid PAGE analysis of products of flexizyme reaction with microhelix RNA under various reaction conditions. Lane 1: a negative control without any acid substrate; lanes 2–4: reactions in the presence of activated 5-mercapto-norvaline-3,5-dinitrobenzyl ester (Mnv-DBE) with various reaction times (2, 6 and 15 h); lanes 5–7: reactions in the presence of activated 5-[(2-hydroxyethyl)dithio]-norvaline [Mnv(2-Me)-DBE] with various reaction times (2, 6 and 15 h). Bands corresponding to flexizyme, aminoacyl-microhelix RNA and free microhelix RNA were labeled in the figure. Acylation yields were calculated on the basis of the band intensity of aminoacyl-microhelix RNA (A) and free microhelix RNA (M), presented as (A)/[(A)+(M)]. This experiment demonstrates that the optimal reaction time for these acid substrates is 6 h.

orthogonality to all endogenous ARSs, regardless of implanting anticodon sequences, and thus serves as our standard orthogonal tRNAs for the reprogramming of elongation codons.

The methodology of genetic code reprogramming also allows us to reprogram the initiation codon[13]. Our extensive studies using a methionine-removed FIT system have shown that a wide variety of non-methionine initiators can be used to initiate translation[12,13,17]. The initiators include proteinogenic L-amino acids, nonproteinogenic D-amino acids, *N*-acyl and *N*-peptidyl amino acids, all of which could be the substrate for flexizymes. As the initiation reprogramming yields peptides with designated N-terminal modifications, a wide array of applications such as N-terminal fluorescent/biotin labeling or peptide cyclization can be readily achieved (see ANTICIPATED RESULTS section). The initiator tRNA used for reprogramming is $tRNA^{fMetE}_{CAU}$, derived from *E. coli* initiator tRNA with a single mutation of C1G and no base modifications (see ref. 29). It should be noted that this $tRNA^{fMetE}_{CAU}$ is charged with Met, followed by formylation by endogenous enzymes, unlike the elongator $tRNA^{AsnE2}_{NNN}$. However, omitting Met is sufficient enough to deplete the background initiation by fMet.

DNA templates for translation. The template DNA for translation requires the following elements in the 5′ to 3′ order: a T7 promoter, a GGG triplet (enhancer of transcription efficiency), an epsilon sequence (an AU-rich bacterial translation enhancer element originally found in the nontranslated region of the T7 phage gene)[30], a Shine-Dalgano sequence (a purine-rich sequence that functions as a ribosomal binding site), a start codon (ATG), a peptide coding sequence, a stop codon (TAA or TGA) and an additional 6- to 10-bp sequence downstream of the stop codon (**Fig. 1**). The template DNA can be synthesized by two-step or three-step PCR as described in reference 29. If necessary, plasmid DNA can also be used as a template instead of the PCR product. It should be noted that when plasmid DNA is used a T7 terminator sequence should be placed at a downstream region of the stop codon. As the FIT system includes T7 RNA polymerase, i.e., a transcription-translation-coupled system, the DNA template is *in situ* transcribed into mRNA that is translated to the designated peptide.

Preparation of the FIT system and expression of nonstandard peptides. The translation system used in the FIT system consists of the essential components[31,32] used for translation and transcription (e.g., ribosomes, initiation factors, elongation factors, release factors, tRNAs, amino acids, ARSs, T7 RNA polymerase, NTPs and template DNA). Purified *E. coli* total tRNA, some proteins (e.g., creatine kinase and myokinase) and low-molecular-weight compounds can be obtained from commercial sources. Other individual protein factors are generally overexpressed in *E. coli*, with appropriate tags such as polyhistidine, and purified by affinity chromatography according to standard methodologies[33,34]. However, tags are not essential for the reconstitution of the FIT system; therefore, a tag can be removed by an appropriate protease when a specific digestion site is embedded in the linker region between the tag and protein factors.

To facilitate the reconstitution of various FIT systems, three solutions are prepared separately; one—the TS solution—is composed of total tRNAs and small molecules, including buffer, nucleotide triphosphates and amino acids; the second—the RP solution—contains ribosomes and essential protein factors; the third—the ARS solution—consists of ARSs. Expression of the desired nonstandard peptide containing nonproteinogenic amino acids is done using a translation system consisting of the mixture of the TS, RP and ARS solutions, wherein the designated amino acids and/or ARSs are simply excluded and desired Xaa-tRNAs are supplied. Note that these three solutions are customized to match the components, depending on the design of the reprogrammed genetic code; for instance, methionyl-tRNA formyltransferase (MTF) and its formyl-donor substrate (10-formyl-5,6,7,8-tetrahydrofolic acid) can be removed for N-terminal modifications, or certain enzymes (e.g., peptide deformylase and methionine aminopeptidase) can be added to remove the *N*-formyl-methionine from the expressed peptide *in situ*. Note that the translation mixture of the FIT system also contains flexizymes along with Xaa-tRNAs. Xaa-tRNA synthesized by flexizyme is prepared by simple ethanol precipitation and no further purification is required. The optimal concentration of Xaa-tRNAs in the translation mixture varies depending on the type of Xaa, but 50–100 μM of each Xaa-tRNA is generally used in our laboratory. If the expression level monitored by radiolabeled peptides migrated in tricine-SDS-PAGE turned out to be unsatisfactory, a further increase in the concentration of Xaa-tRNA would improve the yield of product peptides. We have confirmed that a total concentration up to 600-μM Xaa-tRNAs does not disturb the translation when aa-tRNAs are well desalted by the procedure described in this protocol.

Analysis and evaluation of expressed peptides in the FIT system. Peptides expressed using the FIT system can be analyzed in various ways. For quantitative analysis, peptides are expressed in the presence of an appropriate radioisotope-labeled amino acid, and the samples taken from the translation mixture are directly analyzed by tricine-SDS-PAGE[35]. The radioactivity of the peptide

band is quantified in comparison with known amounts of a radioisotope-labeled amino acid, which reliably estimates the quantity of peptide expressed. Desalting of the translation product using a solid-phase extraction (SPE) column followed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry is sufficient to qualitatively analyze the peptide identity[36,37].

Peptide synthesis using the FIT system is readily coupled with various functional assay protocols[19,24]. The FIT translation mixture containing the expressed nonstandard peptides can be directly added to a variety of assay formats (e.g., biochemical assays, cell assays) without further purification to evaluate their bioactivity or inhibitory activity, as it does not contain organic solvents or chemical reagents that can affect the assay method. This feature allows parallel synthesis of various peptides and rapid screening from the resulting library to discover a new class of nonstandard peptides with the desired bioactivity.

MATERIALS

REAGENTS

- HEPES (Dojindo Laboratories, cat. no. 342-01375)
- Potassium hydroxide (Nacalai tesque, cat. no. 28616-45) **! CAUTION** It is corrosive and an irritant. Wear gloves and protective glasses.
- Magnesium chloride hexahydrate (Nacalai tesque, cat. no. 20909-55)
- DMSO-d_6 (Cambridge Isotope Laboratories, cat. no. DLM-10TB)
- Sodium acetate buffer solution (3 mol l^{-1}, pH 5.2; Nacalai tesque, cat. no. 31138-31)
- Ethanol (Nacalai tesque, cat. no. 14713-95) **! CAUTION** It is flammable.
- Formamide (Wako Pure Chemical Industries, cat. no. 068-00426)
- 3′,3″,5′,5″-tetrabromophenolsulfonphthalein sodium salt (BPB; Sigma, cat. no. B-8026)
- Acrylamide/Bis mixed solution (19:1, 40% (wt/vol)); nuclease and protease tested (Nacalai tesque, cat. no. 00857-55) **! CAUTION** It may cause nervous system damage and is a suspected carcinogen. Wear gloves.
- Urea (Nacalai tesque, cat. no. 35905-64)
- Ammonium peroxodisulfate (APS; Wako Pure Chemical Industries, cat. no. 016-08021) **! CAUTION** It is a strong oxidizer and an irritant.
- *N,N,N′,N′*-Tetramethylethylenediamine (TEMED; Nacalai tesque, cat. no. 33401-72) **! CAUTION** It is flammable and an irritant.
- Tris(hydroxymethyl)aminomethane (Tris), nuclease and protease tested (Nacalai tesque, cat. no. 35434-34)
- Boric acid (Nacalai tesque, cat. no. 05215-05)
- 0.5 mol/l-EDTA solution (pH 8.0) (Nacalai tesque, cat. no. 14347-21)
- Ethidium bromide solution (10 mg ml^{-1}; Nacalai tesque, cat. no. 14631-94) **! CAUTION** It is a mutagen and an irritant. Wear gloves.
- L-(+)-Isoleucine (Ile, Nacalai tesque, cat. no. 20330-02)
- L-(−)-Phenylalanine (Nacalai tesque, cat. no. 26910-22)
- L-Glutamic acid (Nacalai tesque, cat. no. 16911-22)
- L-Aspartic acid (Nacalai tesque, cat. no. 03503-72)
- L-Arginine (Nacalai tesque, cat. no. 03321-52)
- L-Lysine monohydrochloride (Nacalai tesque, cat. no. 20809-52)
- L-Tryptophan (Nacalai tesque, cat. no. 35607-32)
- L-Leucine (Nacalai tesque, cat. no. 20327-62)
- L-Asparagine monohydrate (Nacalai tesque, cat. no. 03427-82)
- L-Methionine (Nacalai tesque, cat. no. 21719-02)
- L-Glutamine (Nacalai tesque, cat. no. 16919-42)
- L-Valine (Nacalai tesque, cat. no. 36108-42)
- L-Cysteine (Nacalai tesque, cat. no. 10309-12)
- L-Histidine monohydrochloride monohydrate (Nacalai tesque, cat. no. 18119-62)
- L-Threonine (Nacalai tesque, cat. no. 33820-82)
- L-α-Alanine (Nacalai tesque, cat. no. 01115-22)
- Glycine (Nacalai tesque, cat. no. 17109-22)
- L-(−)-Proline (Nacalai tesque, cat. no. 29001-42)
- L-Serine (Nacalai tesque, cat. no. 30608-72)
- L-Tyrosine (Nacalai tesque, cat. no. 35709-02)
- Adenosine 5′-triphosphate disodium salt n-hydrate (Wako Pure Chemical Industries, cat. no. 133-00725)
- CTP sodium salt (Jena Bioscience, cat. no. NU-1011)
- GTP sodium salt (Jena Bioscience, cat. no. NU-1012)
- UTP sodium salt (Jena Bioscience, cat. no. NU-1013)
- Potassium acetate (Nacalai tesque, cat. no. 28405-05)
- Magnesium acetate tetrahydrate (Nacalai tesque, cat. no. 20821-85)
- Creatine phosphate (Roche, cat. no. 621722)
- Folinic acid calcium salt, pentahydrate (Acros Organics, cat. no. 23031)
- Spermidine (Nacalai tesque, cat. no. 32108-04) **! CAUTION** It is corrosive.
- *E. coli* total tRNA (Roche, cat. no. 109550)
- Creatine kinase (Roche, cat. no. 736988)
- Myokinase (Sigma, cat. no. M5520)
- Acetonitrile (Nacalai tesque, cat. no. 00405-23) **! CAUTION** It is flammable and an irritant.
- Acetic acid (Kanto chemical, cat. no. 01021-00) **! CAUTION** It is corrosive and an irritant.
- α-Cyano-4-hydroxy-cinnamic acid (Bruker Daltonics, cat. no. 201344)
- RNase-free water (generated by an ultrapure water system)

EQUIPMENT

- Ultrapure water system (Sartorius, arium 611UV)
- Vortex mixer (TM-2000, Iwaki)
- Heat block (Nissin, ND-MO1)
- Refrigerated microcentrifuge (TOMY, MX-305)
- Mini-gel apparatus (Bio-Rad, Mini-PROTEAN 3)
- Power supply for electrophoresis (Bio-Rad, PowerPac Basic)
- Fluorescent gel scanner (FLA-5100, Fuji)
- Low binding tubes (0.6 ml; BM Bio, BM4006)
- Air incubator (Isuzu Seisakusho, FR-115S)
- MALDI-TOF mass spectrometer (Bruker Daltonics, Autoflex II)
- SPE C-tip (Nikkyo Technos)
- Injection syringe (10 ml; Terumo)
- Benchtop centrifuge (Nikkyo Technos)

REAGENT SETUP

HEPES-KOH buffer (500 mM; pH 7.5) Mix 4.77 g of HEPES and 2.5 ml of 2 M KOH, then add water up to 35 ml. Dilute a small portion of the solution to prepare a 50 mM HEPES solution, and measure the pH at 4 °C. Add 2 M KOH into the original HEPES solution until the pH of the diluted solution at 4 °C reaches 7.5. After pH adjustment, add water up to 40 ml into the original HEPES solution. Store it in aliquots at −20 °C (stable for years).

Flexizymes Prepare flexizyme RNAs by the conventional *in vitro* transcription method[38] according to procedures described in reference 29.

Microhelix RNA Prepare it by the conventional *in vitro* transcription method according to procedures described in reference 29.

Acid substrate (25 mM) in DMSO-d_6 Synthesize acid substrate activated with an appropriate active group for flexizyme reaction in two steps from N-Boc-protected amino acids according to the previously reported method[3,10,27]. To a solid of acid substrate, add DMSO-d_6 to prepare a 200 mM stock solution. Store it at −20 °C (it is generally stable for 1 year). To make a 25 mM working solution, mix 10 μl of 200 mM stock solution with 70 μl of DMSO-d_6. The 25 mM working solution can be stored in aliquots at −20 °C (they are generally stable for 1 year). In case the quality of acid substrate stock solution needs to be checked, measure its NMR spectrum.

Sodium acetate (0.3 M, pH 5.2) Mix 4 ml of 3 M sodium acetate (pH 5.2) and 36 ml of water. Can be stored at room temperature (25 °C) for at least 6 months.

Ethanol containing 0.1 M sodium acetate (pH 5.2) Mix 1.33 ml of 3 M sodium acetate (pH 5.2), 28 ml of 70% (vol/vol) ethanol and 10.67 ml of water. Can be stored at room temperature for at least 6 months.

Acid PAGE loading buffer Mix 50 μl of 3 M sodium acetate (pH 5.2), 20 μl of 0.5 M EDTA (pH 8.0), 930 μl of formamide and 8 μl of 2% (wt/vol) BPB. Can be stored at room temperature for at least a year.

APS (10% (wt/vol)) To 1 g of APS add water up to 10 ml. Can be stored at 4 °C for at least 3 months.

Acid-acrylamide gel solution (20% (wt/vol)) Mix 1.8 g of urea, 83 μl of 3 M sodium acetate (pH 5.2) and 2.5 ml of 40% (wt/vol) acrylamide/bisacrylamide (19:1) solution, and add RNase-free water up to 5 ml. Mix gently until urea dissolves completely. Add 50 μl of 10% (wt/vol) APS and 4 μl of TEMED to the solution right before pouring into a mini-gel equipment. Prepare freshly before use.
5× TBE Mix 53.91 g of Tris, 27.51 g of boric acid, and 20 ml of 0.5 M EDTA (pH 8.0), then add water up to 1 liter. Can be stored at room temperature for 3 months.
1× TBE Mix 800 ml of water and 200 ml of 5× TBE. Can be stored at room temperature for at least 6 months.
Ethidium bromide solution for gel staining Mix 10 ml of 1× TBE and 0.5 μl of 10 mg ml^{-1} ethidium bromide. Prepare fresh before use.
tRNAs used for flexizyme reactions Prepare required tRNAs by the conventional *in vitro* transcription method according to procedures described in reference 29.
Template DNA for translation Prepare a DNA duplex coding a desirable peptide as described in reference 29.
Ethanol (70% (vol/vol)) Mix 12 ml of water and 28 ml of ethanol. Can be stored at room temperature for at least 6 months.
***E. coli* ribosome** Purify ribosome from the *E. coli* A19 strain by following a method reported previously[39]. Can be stored at −80 °C for at least 1 year.
Proteins involved in the translation system of the FIT system Express the proteins required for reconstitution of the translation system in *E. coli* and purify them according to standard His-tag or other appropriate methodologies[33,34]. The purified components involved in the translation system are as follows: *E. coli* ribosome, IF1, IF2, IF3, EF-G, EF-Tu, EF-Ts, RF2, RF3, RRF, AlaRS, ArgRS, AsnRS, AspRS, CysRS, GluRS, GlnRS, GlyRS, HisRS, IleRS, LeuRS, LysRS, MetRS, PheRS, ProRS, SerRS, ThrRS, TrpRS, TyrRS, ValRS, MTF, T7 RNA polymerase, inorganic pyrophosphatase and nucleotide diphosphate kinase. Can be stored at −80 °C for at least 1 year.
10-formyl-5,6,7,8-tetrahydrofolic acid solution Synthesize 10-formyl-5, 6,7,8-tetrahydrofolic acid from folinic acid according to the previously reported method[40]. In the FIT system in which the initiation reprogramming is performed, this molecule as well as MTF can be excluded for simplicity.
TS solution (10×) This solution is prepared using 500 mM HEPES-KOH (pH 7.6), 1 M potassium acetate, 120 mM magnesium acetate, 20 mM ATP, 20 mM GTP, 10 mM CTP, 10 mM UTP, 200 mM creatine phosphate, 1 mM 10-formyl-5,6,7,8-tetrahydrofolic acid, 20 mM spermidine, 10 mM DTT and 15-mg ml^{-1} *E. coli* total tRNA. Use and store in low binding tubes in aliquots at −20 °C. Can be stored at −80 °C for at least 6 months.
RP solution (10×) This solution comprises 3 mM magnesium acetate, 12 μM *E. coli* ribosome, 6 μM MTF, 27 μM IF1, 4 μM IF2, 15 μM IF3, 2.6 μM EF-G, 100 μM EF-Tu, 6.6 μM EF-Ts, 2.5 μM RF2, 1.7 μM RF3, 5 μM RRF, 40 μg ml^{-1} creatine kinase, 30 μg ml^{-1} myokinase, 1 μM inorganic pyrophosphatase, 1 μM nucleotide diphosphate kinase and 1 μM T7 RNA polymerase. Use and store in low-binding tubes in aliquots at −80 °C. Can be stored at −80 °C for at least 6 months.
ARS solution (10×) Mix the necessary ARSs for the objective genetic code reprogramming with the following concentrations: 7.3 μM AlaRS, 0.3 μM ArgRS, 3.8 μM AsnRS, 1.3 μM AspRS, 0.2 μM CysRS, 0.6 μM GlnRS, 2.3 μM GluRS, 0.9 μM GlyRS, 0.2 μM HisRS, 4.0 μM IleRS, 0.4 μM LeuRS, 1.1 μM LysRS, 0.3 μM MetRS, 6.8 μM PheRS, 1.6 μM ProRS, 0.4 μM SerRS, 0.9 μM ThrRS, 0.3 μM TrpRS, 0.2 μM TyrRS and 0.2 μM ValRS. Can be stored at −80 °C for at least 6 months.
Amino acid solutions (5 mM each) Mix proteinogenic amino acids, except for the ones to be replaced with nonproteinogenic amino acids in the objective reprogrammed genetic code, to prepare a 5 mM stock solution. Can be stored at −20 °C for at least 6 months.
SPE solution A Mix 800 μl of acetonitrile, 195 μl of water and 5 μl of acetic acid. Store at room temperature. Can be stored at room temperature for at least 1 month.
SPE solution B Mix 40 μl of acetonitrile, 955 μl of water and 5 μl of acetic acid. Store at room temperature. Can be stored at room temperature for at least 1 month.
Matrix solution for MALDI-TOF MS Prepare 50% saturated α-cyano-4-hydroxy-cinnamic acid solution in SPE solution A. Prepare this solution freshly for each experiment.

EQUIPMENT SETUP

Acid-polyacrylamide gel electrophoresis Assemble gel plates (Bio-Rad, Mini-PROTEAN 3) and pour acid-acrylamide gel solution (20% (wt/vol)) into the gel plates. Store at room temperature until the gel solidifies. Use 50 mM Sodium acetate (pH 5.2) as a running buffer. Prepare gels freshly for each experiment.

PROCEDURE

Optimization of conditions for flexizyme reaction ● TIMING 6 h

1| Mix 1 μl of 500 mM HEPES-KOH buffer (pH 7.5), 1 μl of 250 μM flexizyme of choice (see below) and 1 μl of 250 μM microhelix RNA with 3 μl of RNase-free water.
▲ CRITICAL STEP An appropriate flexizyme should be used depending on the side chain and active group of acid substrates (see Experimental design and **Table 1**). Use of buffers at higher pH, such as HEPES-KOH (pH 8.0) and Bicine-KOH (pH 9.0), may improve the yield of acylation in some cases.
▲ CRITICAL STEP All of the following steps should be carried out in an RNase-free manner. Use RNase-free tubes, pipettes, pipette tips and water. Wear gloves at all times.

2| Heat the sample at 95 °C for 2 min, then slowly cool it at room temperature over 5 min.

3| Add 2 μl of 3 M $MgCl_2$ into the sample, then incubate it at room temperature for 5 min followed by incubation on ice for 3 min.

4| Add 2 μl of 25 mM acid substrate in DMSO-d_6 into the sample and mix well.

5| Incubate the acylation reaction mixtures on ice for several hours.
▲ CRITICAL STEP The ideal reaction time strongly varies depending on acid substrates. Perform several reactions with various reaction times to find the optimal reaction time (see Experimental design and **Table 1**).

6| Add 40 μl of 0.3 M sodium acetate (pH 5.2) and 100 μl of ethanol into the reaction mixtures to quench the acylation reaction.

7| Centrifuge the samples at 15,000*g* for 15 min at 25 °C. Thereafter, remove the supernatant completely.

8| Add 4 μl of 10 mM sodium acetate (pH 5.2) to the tubes and dissolve the RNA pellets quickly.
▲ **CRITICAL STEP** Acyl-tRNAs may be unstable; accordingly, dissolve the RNA pellet right before applying to an acid-polyacrylamide gel. After adding 10 mM sodium acetate (pH 5.2), perform the following steps promptly.

9| Add 12 μl of acid PAGE loading buffer to the samples and mix well.

10| Apply 2 μl each of the resulting samples onto an acid-polyacrylamide gel and run it at 120 V for 2.5 h.

11| Wash the gel with 50 ml of 1× TBE by gently shaking for 10 min.

12| Stain the gel with 10 ml of ethidium bromide gel-staining solution by gently shaking for 10 min.
! CAUTION Ethidium bromide may be a mutagen. The used gel-staining solution and washing solution in the following steps should be disposed of in a proper manner.

13| Wash the gel briefly with 50 ml of RNase-free water.

14| Wash the gel with 50 ml of 1× TBE by gently shaking for 5 min.

15| Scan the gel image with a fluorescent gel scanner.
? TROUBLESHOOTING

16| Quantify the bands corresponding to free and acylated microhelix RNA to determine the yield of acylation (see **Fig. 3** for an example of acid PAGE analysis).
? TROUBLESHOOTING

Acylation of tRNAs by flexizyme ● TIMING 3–30h

17| Mix 2 μl of 500 mM HEPES-KOH buffer (pH 7.5), 2 μl of 250 μM flexizyme of choice (see below) and 2 μl of 250 μM tRNA of choice (see ▲ **CRITICAL STEP** below) with 6 μl of RNase-free water.
▲ **CRITICAL STEP** An appropriate flexizyme should be chosen depending on the side chain and active group of acid substrates (see Experimental design and **Table 1**). The body sequence and anticodon of the tRNA should be chosen for appropriately reprogramming the codon.

18| Repeat Steps 2–5 using double the volumes specified in those steps in all cases.
▲ **CRITICAL STEP** Proper reaction time strongly varies depending on acid substrates. We recommend optimizing the reaction conditions by using microhelix RNA and acid PAGE analysis (see Steps 1–16), and then use the optimal conditions for the synthesis of desired acyl-tRNA.

19| Repeat Steps 6 and 7 using double the volumes specified in those steps in all cases.

20| Add 50 μl of 70% (vol/vol) ethanol containing 0.1 M sodium acetate (pH 5.2) to the tube and vortex the tube well to break the RNA pellet into pieces.
▲ **CRITICAL STEP** Ethanol precipitation and the following washing steps (Steps 20–24) should be carried out at around room temperature, not at a lower temperature, to avoid undesirable precipitation of salts.
▲ **CRITICAL STEP** The washing steps (Steps 20–24) are crucial for the following translation reaction. Carryover of magnesium and sodium ions would decrease the efficiency of translation reaction.

21| Centrifuge the sample at 15,000*g* for 5 min at 25 °C. Thereafter, remove the supernatant completely.

22| Repeat Steps 20 and 21 one more time.

23| Add 50 μl of 70% (vol/vol) ethanol to the tube.

24| Centrifuge the sample at 15,000*g* for 3 min at 25 °C.

25| Open the tube lid and cover it with tissues, then dry the RNA at room temperature for 5 min.
■ **PAUSE POINT** Dried acyl-tRNA pellets can be stored at −80 °C for 1 d. Some of them may be stored for weeks, depending on the structure of amino acids.

Translation of peptides using reprogrammed genetic code ● **TIMING 2 h**
26| Set up the translation reaction (5 μl) as follows:

Component	Amount per reaction
TS solution (10×)	0.5 μl
RP solution (10×)	0.5 μl
ARS solution (10×)	0.5 μl
5 mM each amino acids solution	0.5 μl
DNA template (see ref. 29)	0.2 pmol
Acyl-tRNAs synthesized by flexizyme (from Step 25)	500 pmol each in 1 μl of 1 mM sodium acetate (pH 5.2)
RNase-free water	to 5 μl

27| Incubate the reaction mixture at 37 °C for 1h.
▲ **CRITICAL STEP** Do not add the proteinogenic amino acids the codons of which are reprogrammed by nonproteinogenic acyl-tRNAs.
▲ **CRITICAL STEP** Resuspend the acyl-tRNAs in 1 μl of 1 mM sodium acetate (pH 5.2) just before mixing them with the translation reaction mixture, because they are unstable and easily hydrolyzed in solution (especially under alkaline conditions).

Analysis of the resulting peptides by MALDI-TOF mass spectrometry ● **TIMING 1 h**
28| Add 15 μl of SPE solution A to the SPE C-tip column for preconditioning. The solution should be loaded from the top of the tip using a pipette. Spin the C-tip for 15 s using a benchtop centrifuge.
▲ **CRITICAL STEP** Be careful not to let the acetonitrile vaporize during manipulation of solution A.

29| Add 15 μl of SPE solution B to the C-tip column. Spin the C-tip for 15 s by benchtop centrifuge.
▲ **CRITICAL STEP** Move on to the next step immediately while the resin is still wet.

30| Add the translation reaction mixture from Step 27 to the C-tip. Spin the C-tip for 15 s with a benchtop centrifuge.

31| Add 15 μl of SPE solution B to wash the column. Spin the C-tip for 15 s with a benchtop centrifuge.
▲ **CRITICAL STEP** Wash the wall of the tip well by pipetting.

32| Repeat Step 31.

33| Add 1 μl of MALDI matrix solution, and elute the peptide from the C-tip by pressing the liquid using an air-filled 10-ml plastic syringe. The solution can be directly deposited onto the MALDI target plate.
▲ **CRITICAL STEP** Use freshly prepared matrix solution.

34| Dry the peptide-matrix mixture at room temperature. Let the target plate stand until the crystallization of matrix is complete.

35| Measure the MALDI-TOF mass spectrum[3].
? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
15	Smearing of bands	Contamination with RNase	Be careful not to contaminate reagents and reaction mixture with RNase. Wear gloves when performing experiments. Prepare all reagents freshly when RNase contamination is suspected
16	Low acylation yield	An inappropriate choice of flexizyme matched with the specific active group was used	Choose an appropriate combination of flexizyme and active group for the objective acid, as described in Experimental design
		Very slow acylation	In some acid substrates, particularly those with α-*N*-acyl or bulky side chains, a long incubation time (up to 72 h) may be required
		Low solubility of acid substrate	Increase the final concentration of DMSO up to 40%. Alternatively, change the leaving group of substrate to ABT in combination with aFx
		Low reactivity of acid substrate	Increase the pH of flexizyme reaction from 7.5 up to 9.5. If the yield of Xaa-tRNA is still low, increase the concentration of acid substrate from the generic concentration (5 mM) to 25 or 40 mM. These changes in conditions occasionally improve the poor yield
35	Low signal of the translation products	Inhibition of translation reaction by salts carried over from the acylation reaction to the translation system	Wash acyl-tRNA pellet well according to the procedures described in Steps 24–28
	Misincorporation of undesirable proteinogenic amino acid(s) into the reprogrammed codon(s)	Contamination of a trace amount of amino acid(s) present in the translation factors or ARSs	Further purify the translation factors or ARSs to reduce the trace amount of cognate amino acids as much as possible. If the misincorporation still occurs, use other codons for reassignment of non-proteinogenic amino acids

● TIMING

Steps 1–16, Optimization of conditions for flexizyme reaction: 6h (hands-on time: 2 h)
Steps 17–25, Preparation of acyl-tRNA by flexizyme: 3–30 h (hands-on time: 1 h)
Steps 26 and 27, Translation of peptides using reprogrammed genetic code: 2 h
Steps 28–35, Analysis of the resulting peptides by MALDI-TOF mass spectrometry: 1 h

ANTICIPATED RESULTS

Expression results of a representative cyclic *N*-methyl peptide and a cyclic peptoid-peptide hybrid are illustrated in **Figure 4**. For the cyclic *N*-methyl peptide synthesis, we demonstrated the reprogramming of the AUG initiation codon and three sense codons in elongation, introducing ^{ClAc}Phe and three α-*N*-methyl amino acids (^{Me}Ala, ^{Me}Tyr and ^{Me}Gly), respectively. Translations were performed according to the reprogrammed genetic code using the four corresponding Xaa-tRNAs, where ^{Me}Ala, ^{Me}Tyr and ^{Me}Gly were coded by UCC, CAC and CGC, respectively (**Fig. 4a,c**). For synthesis of the cyclic-peptoid-peptide hybrid, α-*N*-chloroacetyl D-phenylalanine (^{ClAc}D-Phe) and α-*N*-alkyl glycines (*N*-methyl-glycine; *N*-*n*-propyl-glycine, ^{Pr}Gly; *N*-*n*-butyl-glycine, ^{Bu}Gly) were incorporated instead of ^{ClAc}Phe and α-*N*-methyl amino acids, respectively, using the same DNA template but a different reprogrammed genetic code (**Fig. 4b,c**). The resulting N-terminal chloroacetamide group forms a thioether bond intramolecularly with the sulfhydryl group of the cysteine residue in the peptide chain, and yields a cyclic peptide (**Fig. 4d,e**). A single peak of the desired product should be observed in MALDI-TOF mass spectrum as shown in **Figure 4f,g**. Sodium and potassium adducts of the peptide may be detected, if the washing of SPE resin is not enough. Although protein and RNA components derived from the translation system cannot be removed by SPE purification, their peaks are generally not found in MALDI-TOF MS because of the difference of the mass range.

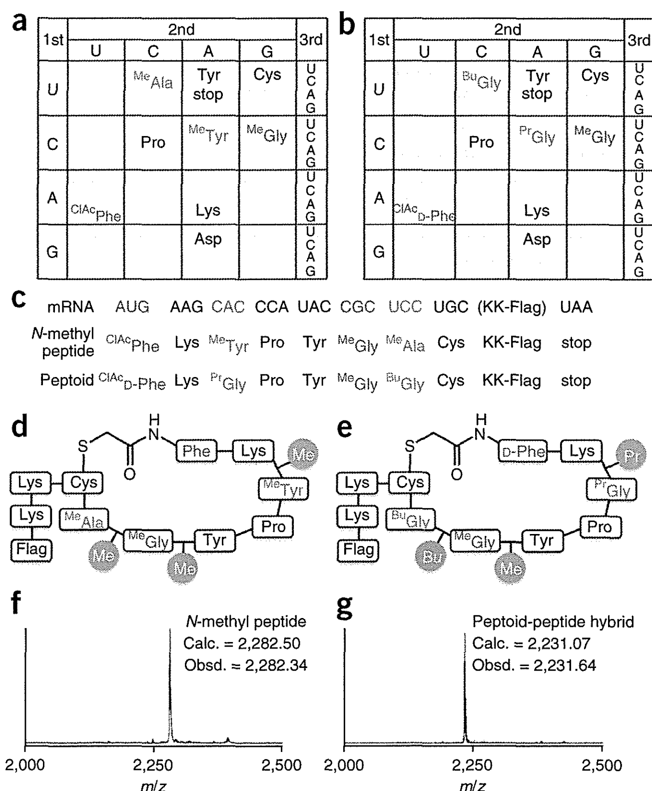

Figure 4 | Typical results of cyclic *N*-alkyl peptide syntheses by genetic code reprogramming. (**a**,**b**) Reprogrammed genetic code tables used for cyclic *N*-methyl peptide synthesis (**a**) and cyclic peptoid-peptide hybrid synthesis (**b**). Nonproteinogenic amino acids assigned in the reprogrammed codons are shown in colored letters. (**c**) Sequences of mRNA template and linear peptide/peptoid translation products. The KK-Flag in parenthesis indicates the RNA sequence encoding a KK-Flag peptide (KK-DYKDDDDK). (**d**,**e**) Schematic structures of cyclic *N*-methyl-peptide (**d**) and cyclic peptoid-peptide hybrid (**e**) synthesized by the FIT system via spontaneous post-translational cyclization. (**f**,**g**) MALDI-TOF mass spectra of cyclic *N*-methyl-peptide (**f**) and cyclic peptoid-peptide hybrid (**g**). The calculated mass (Calc.) and observed mass (Obsd.) for proton-adduct ions ($[M+H]^+$) are shown in each spectrum.

To estimate the amount of the produced peptide, a FIT translation system in which one of the amino acids is replaced with the radioisotope-labeled equivalent (e.g., replacing Asp with [^{14}C]) can be used for the expression of radioisotope-labeled peptides followed by quantitative analysis by tricine-SDS-PAGE. A typical yield of translation products using the FIT system is in the range of 5–250 pmol in a 5-μl translation. However, it can vary depending on the type and number of nonproteinogenic amino acids introduced into the peptide.

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AUTHOR CONTRIBUTIONS Y.G. developed the methods presented in this study. Y.G. and T.K. validated the protocol, wrote the article and prepared the figures. H.S. supervised all the work and prepared the final version of the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare competing financial interests (see the HTML version of this article for details).

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1. Forster, A.C. *et al.* Programming peptidomimetic syntheses by translating genetic codes designed *de novo*. *Proc. Natl. Acad. Sci. USA* **100**, 6353–6357 (2003).
2. Josephson, K., Hartman, M.C. & Szostak, J.W. Ribosomal synthesis of unnatural peptides. *J. Am. Chem. Soc.* **127**, 11727–11735 (2005).
3. Murakami, H., Ohta, A., Ashigai, H. & Suga, H. A highly flexible tRNA acylation method for non-natural polypeptide synthesis. *Nat. Methods* **3**, 357–359 (2006).
4. Hecht, S.M., Alford, B.L., Kuroda, Y. & Kitano, S. 'Chemical aminoacylation' of tRNA's. *J. Biol. Chem.* **253**, 4517–4520 (1978).
5. Wang, L., Xie, J. & Schultz, P.G. Expanding the genetic code. *Annu. Rev. Biophys. Biomol. Struct.* **35**, 225–249 (2006).
6. Noren, C.J., Anthony-Cahill, S.J., Griffith, M.C. & Schultz, P.G. A general method for site-specific incorporation of unnatural amino acids into proteins. *Science* **244**, 182–188 (1989).
7. Hohsaka, T., Ashizuka, Y., Murakami, H. & Sisido, M. Incorporation of nonnatural amino acids into streptavidin through *in vitro* frame-shift suppression. *J. Am. Chem. Soc.* **118**, 9778–9779 (1996).
8. Cropp, T.A., Anderson, J.C. & Chin, J.W. Reprogramming the amino-acid substrate specificity of orthogonal aminoacyl-tRNA synthetases to expand the genetic code of eukaryotic cells. *Nat. Protoc.* **2**, 2590–2600 (2007).
9. Murakami, H., Saito, H. & Suga, H. A versatile tRNA aminoacylation catalyst based on RNA. *Chem. Biol.* **10**, 655–662 (2003).
10. Niwa, N., Yamagishi, Y., Murakami, H. & Suga, H. A flexizyme that selectively charges amino acids activated by a water-friendly leaving group. *Bioorg. Med. Chem. Lett.* **19**, 3892–3894 (2009).
11. Sako, Y., Morimoto, J., Murakami, H. & Suga, H. Ribosomal synthesis of bicyclic peptides via two orthogonal inter-side-chain reactions. *J. Am. Chem. Soc.* **130**, 7232–7234 (2008).
12. Goto, Y., Murakami, H. & Suga, H. Initiating translation with D-amino acids. *RNA* **14**, 1390–1398 (2008).
13. Goto, Y. *et al.* Reprogramming the translation initiation for the synthesis of physiologically stable cyclic peptides. *ACS Chem. Biol.* **3**, 120–129 (2008).
14. Kawakami, T., Murakami, H. & Suga, H. Messenger RNA-programmed incorporation of multiple N-methyl-amino acids into linear and cyclic peptides. *Chem. Biol.* **15**, 32–42 (2008).
15. Kawakami, T., Murakami, H. & Suga, H. Ribosomal synthesis of polypeptoids and peptoid-peptide hybrids. *J. Am. Chem. Soc.* **130**, 16861–16863 (2008).
16. Ohta, A., Murakami, H., Higashimura, E. & Suga, H. Synthesis of polyester by means of genetic code reprogramming. *Chem. Biol.* **14**, 1315–1322 (2007).
17. Goto, Y. & Suga, H. Translation initiation with initiator tRNA charged with exotic peptides. *J. Am. Chem. Soc.* **131**, 5040–5041 (2009).
18. Xiao, H., Murakami, H., Suga, H. & Ferre-D'Amare, A.R. Structural basis of specific tRNA aminoacylation by a small *in vitro* selected ribozyme. *Nature* **454**, 358–361 (2008).
19. Kawakami, T. *et al.* Diverse backbone-cyclized peptides via codon reprogramming. *Nat. Chem. Biol.* **5**, 888–890 (2009).
20. Hornbogen, T. & Zocher, R. Biosynthesis of N-methylated peptides in fungi. In *Handbook of Industrial Mycology, Mycology Series v. 22* (ed., Zhiqiang, A.) 449–477 (Marcel Dekker, 2005).

21. Patch, J.A. & Barron, A.E. Mimicry of bioactive peptides via non-natural, sequence-specific peptidomimetic oligomers. *Curr. Opin. Chem. Biol.* **6**, 872–877 (2002).
22. Sagan, S., Karoyan, P., Lequin, O., Chassaing, G. & Lavielle, S. N- and C alpha-methylation in biologically active peptides: synthesis, structural and functional aspects. *Curr. Med. Chem.* **11**, 2799–2822 (2004).
23. Kwon, Y.U. & Kodadek, T. Quantitative evaluation of the relative cell permeability of peptoids and peptides. *J. Am. Chem. Soc.* **129**, 1508–1509 (2007).
24. Sako, Y., Goto, Y., Murakami, H. & Suga, H. Ribosomal synthesis of peptidase-resistant peptides closed by a nonreducible inter-side-chain bond. *ACS Chem. Biol.* **3**, 241–249 (2008).
25. Martinis, S.A. & Schimmel, P. Enzymatic aminoacylation of sequence-specific RNA minihelices and hybrid duplexes with methionine. *Proc. Natl. Acad. Sci. USA* **89**, 65–69 (1992).
26. Putz, J. *et al.* Rapid selection of aminoacyl-tRNAs based on biotinylation of alpha-NH2 group of charged amino acids. *Nucleic Acids Res.* **25**, 1862–1863 (1997).
27. Saito, H., Kourouklis, D. & Suga, H. An *in vitro* evolved precursor tRNA with aminoacylation activity. *EMBO J.* **20**, 1797–1806 (2001).
28. Hopper, A.K. & Phizicky, E.M. tRNA transfers to the limelight. *Genes Dev.* **17**, 162–180 (2003).
29. Goto, Y., Katoh, T. & Suga, H. Preparation of materials for flexizyme reactions and genetic code reprogramming. *Protoc. Exchange.* doi:10.1038/protex.2011.209 (2011).
30. Olins, P.O., Devine, C.S., Rangwala, S.H. & Kavka, K.S. The T7 phage gene 10 leader Rna, a ribosome-binding site that dramatically enhances the expression of foreign genes in *Escherichia coli*. *Gene* **73**, 227–235 (1988).
31. Kung, H.F. *et al.* DNA-directed *in vitro* synthesis of beta-galactosidase. Studies with purified factors. *J. Biol. Chem.* **252**, 6889–6894 (1977).
32. Shimizu, Y. *et al.* Cell-free translation reconstituted with purified components. *Nat. Biotechnol.* **19**, 751–755 (2001).
33. Charlton, A. & Zachariou, M. Immobilized metal ion affinity chromatography of histidine-tagged fusion proteins. *Methods Mol. Biol.* **421**, 137–149 (2008).
34. Block, H. *et al.* Immobilized-metal affinity chromatography (IMAC): a review. *Meth. Enzymol.* **463**, 439–473 (2009).
35. Schagger, H. Tricine-SDS-PAGE. *Nat. Protoc.* **1**, 16–22 (2006).
36. Shukla, A. & Majors, R.E. Micropipette tip-based sample preparation for bioanalysis. *LCGC Eur.* **18**, 650 (2005).
37. Rappsilber, J., Ishihama, Y. & Mann, M. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* **75**, 663–670 (2003).
38. Milligan, J.F., Groebe, D.R., Witherell, G.W. & Uhlenbeck, O.C. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.* **15**, 8783–8798 (1987).
39. Clemons, W.M. Jr. *et al.* Crystal structure of the 30 S ribosomal subunit from *Thermus thermophilus*: purification, crystallization and structure determination. *J. Mol. Biol.* **310**, 827–843 (2001).
40. Baggott, J.E. *et al.* Cofactor role for 10-formyldihydrofolic acid. *Biochem. J.* **308** (Part 3): 1031–1036 (1995).

Peptide Cyclization

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Synthesis of the Backbone Cyclic Peptide Sunflower Trypsin Inhibitor-1 Promoted by the Induced Peptidyl-tRNA Drop-off**

Taek Jin Kang, Yuuki Hayashi, and Hiroaki Suga**

Ribosomes occasionally stall on mRNA in the middle of translation and one of the mechanisms to rescue the ribosome involves the drop-off of peptidyl-tRNA from the P site of the stalled ribosome.[1] Although peptidyl-tRNA drop-off can, in principle, occur at any position during the translational elongations,[2] the slower rate of the termination step relative to the elongation step can result in the stalling of the ribosome at the last sense codon, which ultimately leads to increased rate of peptidyl-tRNA drop-off.[3] This process presents the possibility for the peptidyl-tRNA drop-off event to be artificially induced under the appropriate conditions. Buckingham and co-workers indeed showed that specific peptidyl-tRNA species could be released from the P site of ribosomes that were paused at the last codon of the A site.[4] Specifically, when an mRNA template coding for fMet-Phe-Leu (fMet = formylated methionine; fM) having a UAA stop codon was translated in an in vitro system, which lacked release factors, fMet-Phe-Leu-$tRNA^{Leu}$ was released from the ribosome. Also the same fMet-Phe-Leu-$tRNA^{Leu}$ drop-off event was observed when an mRNA coding for fMet-Phe-Leu-Ile was translated in an in vitro translation system lacking Ile-$tRNA^{Ile}$. This observation demonstrates that the peptidyl-tRNA drop-off can be artificially induced by an appropriate manipulation of the translation conditions. Although the outcome of the above strategy is the termination of the translation, we postulated that this experimental strategy could be extended by exploiting the unique ester bond in the resulting peptidyl-tRNA. In the course of our studies on reprogramming the genetic code, we have also realized that peptidyl-tRNA drop-off might be induced by the use of a specially reconstituted translation system, from which one of the proteinogenic amino acids is omitted to generate a "vacant codon". Significantly, this approach would allow designation of the site of ribosome stalling, and therefore control peptidyl-tRNA drop-off in a site-specific manner. Herein we report proof-of-concept experiments in which specific peptidyl-tRNA drop-off is brought about by creating a vacant codon. In particular, the drop-off of Pep-Cys-Pro-$tRNA^{Pro}$ (Pep = peptide) results in a series of steps leading to the conversion of the C terminus of the peptide into a thioester structure, which is key to the formation of a backbone-cyclic peptide.

A general scheme for the preparation of backbone cyclic peptides using peptidyl-tRNA drop-off is given in Figure 1. In the typical translation system, the ribosome encounters the His CAC codon, for instance His-$tRNA^{His}{}_{GUG}$, and the peptide

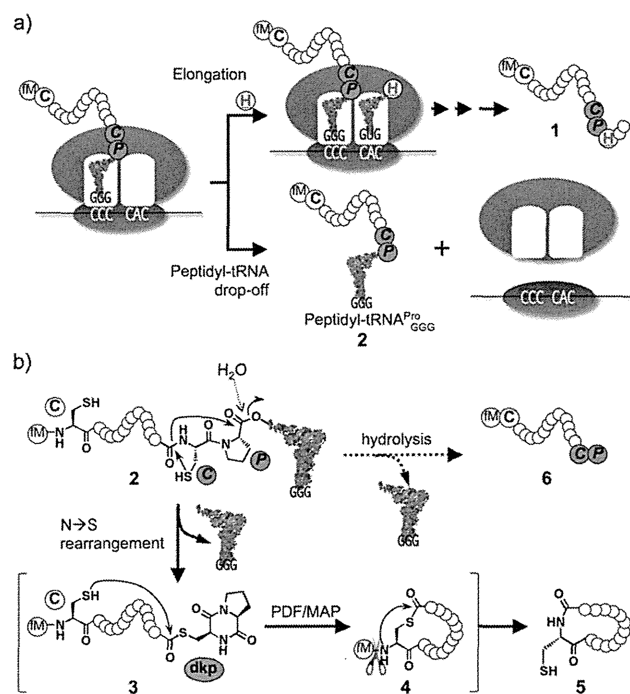

Figure 1. Ribosomal expression of backbone cyclic peptides through the programmed peptidyl-tRNA drop-off. a) Induced peptidyl-tRNA drop-off at the predetermined site. In this example a codon for His (CAC) is used as a vacant codon for the site of peptidyl-tRNA drop-off. b) Intramolecular rearrangements occurring in the released peptidyl-tRNA.

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chain is elongated by incorporating His to yield the full-length peptide **1** (Figure 1 a). We postulated that if His were omitted from the translation components, the ribosome would stall at this vacant His codon and induce the peptidyl-tRNA drop-off (Figure 1 a). If this vacant codon was placed right after the Cys-Pro sequence, the ribosome would stall at the Pro codon of the A site, thus resulting in the release of Pep-Cys-Pro-$tRNA^{Pro}{}_{GGG}$ (**2**, Figure 1). Importantly, it can be envisioned that the ester bond in Pro-(3′-O)-adenosyl76 of Pep-Cys-Pro-$tRNA^{Pro}{}_{GGG}$ is analogous to that in Pro-glycolic acid (^{HO}G) that was generated by the codon reassignment of CAC to ^{HO}G, as we reported recently.[5] Therefore we can expect an intramolecular rearrangement in which an non-enzymatic acyl shift from the nitrogen atom to the sulfur atom affords the diketopiperadine thioester (dkp thioester; **3**) with the concomitant release of $tRNA^{Pro}{}_{GGG}$ (Figure 1 b). This transformation would be driven by the formation of the ring in dkp and would compete with the hydrolysis of the Pro-$tRNA^{Pro}{}_{GGG}$ ester bond to give **6** (Figure 1 b). The dkp thioester formation could promote an additional intramolecular rearrangement involving the thiol group of another Cys to afford the macrothiolactone **4** (Figure 1 b). Moreover, if the free N terminus of the amino group of this Cys was generated by the enzymatic removal of fMet, an acyl shift from the sulfur atom to the nitrogen atom would generate the backbone cyclic peptide **5** (Figure 1 b). Notably, this sequence recycles $tRNA^{Pro}{}_{GGG}$ instead of sequestering it as peptidyl-tRNA, and synthesizes backbone cyclic peptides without the use of a nonproteinogenic ^{HO}G.

To experimentally prove the above concept, we designed an mRNA template that encoded a 20-mer peptide sequence, in which the 14-mer peptide sequence of sunflower trypsin inhibitor-1 (SFTI-1)[6] was embedded from position 2–15 (Figure 2 a). Codons encoding Cys16-Pro17, His18 (a vacant codon), and Lys19-Lys20 were placed after those encoding SFTI-1. We designed and carried out four reactions to prove the mechanism proposed in Figure 1. When the DNA template was subjected to sequential transcription and translation in the typical translation system, the full-length peptide fMet1-Lys20 (**7**) was expressed (Figure 2 b and c). When the same DNA template was decoded under reducing conditions and in the absence of His a new single peak appeared (Figure 2 d). The *m*/*z* value obtained for a singly charged peptide species was consistent with the calculated value of fMet1–Arg15 minus a water molecule, suggesting that the peak corresponds to macrothiolactone **8** (Figures 2 b and d). Importantly, we observed a very minor peak for the hydrolyzed side-product **9** (Figure 2 d), suggesting that the rearrangement of **10** into **8** via the corresponding dkp thioester was rapid enough to out compete the ester hydrolysis. To establish whether the macrothiolactonization was derived from the released Pep(1–15)-Cys16-Pro17-$tRNA^{Pro}{}_{GGG}$ (**10**; Figure 2 b), we attempted to enzymatically hydrolyze the ester bond between Pro17 and $tRNA^{Pro}{}_{GGG}$ in **10** by using peptidyl-tRNA hydrolase (PTH) in the translation system.[7] When PTH was present in the translation mixture, a new single peak corresponding to the molecular mass of the linear peptide fMet1-Pro17 (**9**) was observed (Figures 2 b and e). This data supports the idea that the peptidyl-tRNA drop-off

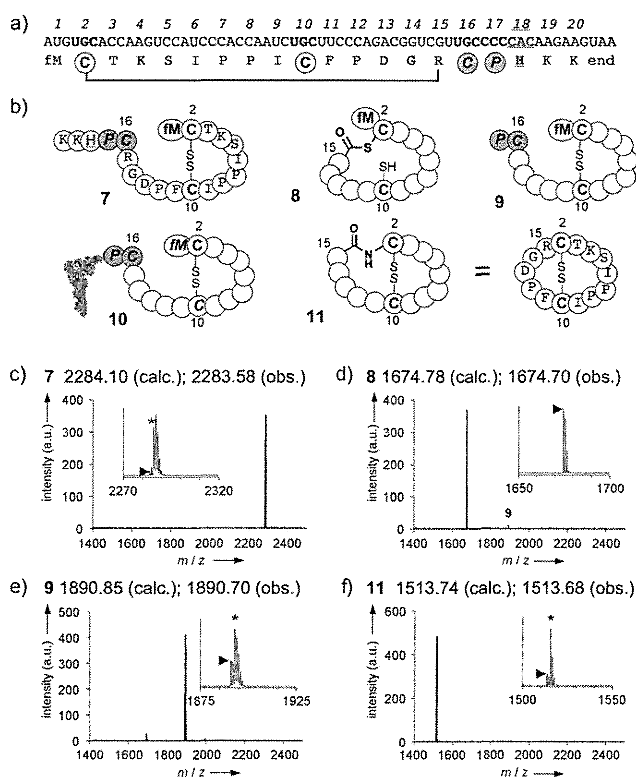

Figure 2. A proof-of-concept study expressing sunflower trypsin inhibitor 1 (SFTI-1) by the induced peptidyl-tRNA drop-off. a) The sequence of mRNA used for the synthesis of SFTI-1. The two internal Cys residues shown in blank circles form the disulfide bond in SFTI-1, whereas Cys and Pro residues in gray circles form the dkp thioester. b) Structure of the key peptide intermediate or the product expressed in this study. c–f) MALDI/TOF analysis of the peptides. For **7**, **9**, and **11** the molecular masses were calculated based on the structure containing a disulfide bond, and the arrow head indicates a set of peaks corresponding to the respective peptide with the *m*/*z* value shown in each spectrum. The peaks representing peptides with the reduced form of the disulfide bond, that is, the two free thiols of the Cys2 and Cys10 residues are indicated by *.

was induced at the designated His codon, and that the released product **10** rearranged into **8** via the dkp thioester intermediate.

In a final reaction, two enzymes, peptide deformylase (PDF) and methionine aminopeptidase (MAP), were included in the translation system lacking a His to promote not only the rearrangement into **8** but also simultaneous removal of fMet1 to expose the N-terminal amino group on Cys2. This system should promote the rearrangement to form the bond resulting in the backbone cyclic peptide. Pleasingly, a new single peak was detected in the mass analysis, and the molecular mass of the purified peptide was in good agreement with the expected mass of the backbone cyclic SFTI-1 **11** (Figures 2 b and f). Overall, peptidyl-tRNA drop-off and the subsequent series of intramolecular rearrangements took place in one pot in 1 hour to give approximately 3.3 $\mu g\,mL^{-1}$ (2.2 $nmol\,mL^{-1}$) of the cyclic SFTI-1 **11** in the reaction solution (see Figure S1 and S2 in the Supporting Information for the quantification and the activity assay, respectively). Notably, SFTI-1 can exist as a bicyclic peptide (**11**(ox) versus

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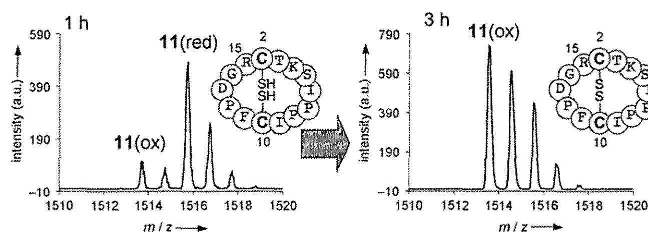

Figure 3. Formation of the disulfide bond in cyclic SFTI-1 by prolonging the translation reaction time. **11**(ox) and **11**(red) indicate oxidized and reduced cyclic SFTI-1, respectively.

11(red); see Figure 3) containing a single disulfide bond. Even though the translation reaction was carried out under reducing conditions using dithiothreitol to promote the series of intramolecular rearrangements from **10** to **11** (Figure 2b), prolonging the reaction time from 1 hour (generic conditions for expression of peptides) to 3 hours was sufficient for the air oxidation of the two Cys side chains to occur to give the disulfide bond (Figure 3). Thus, the simple induced peptidyl-tRNA drop-off methodology enabled us to express **11**(ox) in a one-pot reaction.

We next investigated whether changing the codon assigned to Pro17 or the vacant codon assigned to amino acid 18 would influence the efficiency of the peptidyl-tRNA drop-off event. We found that all four codons, CCN (N = A, U, G, C), coding for Pro gave qualitatively the same result albeit with a higher yield when using CCC rather than the other three codons (see Figure S3 in the Supporting Information). Substitution of the His codon with the Val or Trp codon as a vacant codon also yielded **11**(ox) (see Figure S4 in the Supporting Information).

We have achieved the synthesis of a cyclic peptide by the combination of peptidyl-tRNA drop-off and the spontaneous dkp thioester rearrangement. Although we have previously achieved the same outcome with a site-specific installation of ^{HO}G after the Cys-Pro sequence[5] the peptidyl-tRNA drop-off methodology is simpler and does not require substitution of an amino acid with an α-hydroxy acid. The chemical concept presented here is also applicable to other experimental setups, such as the expression of proteins using an in vitro system that lacks release factors. Studies along this line are underway in our laboratory.

Experimental Section

Experimental details are described in the Supporting Information. Generally, a 2.5 μL scale translation reaction was conducted, using the reconstituted protein synthesis system developed and described previously[5] with 40 nM of DNA template and 1 mM dithiothreitol at 37 °C for 1 hour in the presence or absence of His. In the case of the synthesis of cyclic SFTI-1, the reaction was conducted for 3 h at 37 °C with 1 μM PDF and 3 μM MAP. The peptide was purified and desalted using a C18 ZipTip (Millipore) column and eluted with a solution of acetonitrile/water (1:1) containing 0.1 % of trifluoroacetic acid that was saturated with α-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonics). MALDI-TOF mass analysis was performed using an Autoflex TOF/TOF (Bruker Daltonics) operated in the linear positive mode or reflective positive mode (for monoisotopic analysis) with external calibration (peptide calibration standard II, Bruker Daltonics).

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[1] a) J. Menninger, *J. Biol. Chem.* **1976**, *251*, 3392; b) J. Menninger, *J. Bacteriol.* **1979**, *137*, 694.
[2] a) J. Menninger, *J. Biol. Chem.* **1978**, *253*, 6808; b) V. Heurgué-Hamard, V. Dincbas, R. H. Buckingham, M. Ehrenberg, *EMBO J.* **2000**, *19*, 2701.
[3] J. Menez, V. Heurgue-Hamard, R. H. Buckingham, *Nucleic Acids Res.* **2000**, *28*, 4725.
[4] V. Heurgué-Hamard, R. Karimi, L. Mora, J. MacDougall, C. Leboeuf, G. Grentzmann, M. Ehrenberg, R. H. Buckingham, *EMBO J.* **1998**, *17*, 808.
[5] T. Kawakami, A. Ohta, M. Ohuchi, H. Ashigai, H. Murakami, H. Suga, *Nat. Chem. Biol.* **2009**, *5*, 888.
[6] J. P. Mulvenna, F. M. Foley, D. J. Craik, *J. Biol. Chem.* **2005**, *280*, 32245.
[7] G. Das, U. Varshney, *Microbiology* **2006**, *152*, 2191.

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Ribosome Evolution for Two Artificial Amino Acids in *E. coli*

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Specific incorporation of artificial amino acids into polypeptides offers a useful tool for studying and expanding function. **Neumann et al. (2010)** recently described the evolution of an *E. coli* ribosome that incorporates two artificial amino acids at two designated sites into proteins, in combination with amber codon and a four-base codon.

Expansion of the genetic code has revolutionized the translation machinery-based use of biotechnology for protein or polypeptide expression in which artificial (unnatural) amino acids can be included into the synthesized polypeptide chain (Cornish et al., 1995). The team of Peter G. Schultz has been making a major contribution to the development of such an approach, where an orthogonal pair of tRNA and aminoacyl-tRNA synthetase (ARS) functions in vivo to generate an aminoacyl-tRNA charged with an artificial amino acid (Xie and Schultz, 2006). The amber stop codon, UAG, is generally used for the assignment of an artificial amino acid, since this nonsense codon is most readily suppressible by a tRNA-bearing CUA anticodon ($tRNA_{CUA}$) competing with release factor 1 (RF1) in *E. coli* or other translation systems. Despite the fact that a few dozen of the orthogonal tRNA-ARS pairs are available to date, the expression of desired proteins under the suppression of amber codon occasionally suffers from poor expression efficiency, due to the competing translation termination by RF1 that depends on the incorporation site (e.g., the neighboring codons) or the structure of artificial amino acids. Moreover, the amber suppression strategy is limited to the incorporation of a single type of artificial amino acids into one protein at a time.

The team of Jason W. Chin has made tremendous efforts to evolve 16S ribosomal RNA (rRNA) from the small subunit of *E. coli* ribosome over the last half decade, attempting to circumvent the above restrictions. As a first step, they isolated ribosome variants that recognize a mutant Shine-Dalgano (SD) sequence of mRNA by means of the genetic selection, leading to the development of an orthogonal pair of ribosome and mRNA (O-ribosome and O-mRNA) (Rackham and Chin, 2005). The virtue of developing the O-ribosome—O-mRNA pair is that it facilitates evolution of O-ribosome. Since the wild-type (WT) ribosome and O-ribosome can coexist in *E. coli*, and the O-ribosome does not translate natural mRNAs with the WT SD sequence, even toxic O-ribosome mutants can be evolved without suffering from the fatal outcome. In fact, in their follow-up work, a new O-ribosome mutant, referred to as Ribo-X, was identified and shown to have a unique function, as RF1 was less efficient in termination of the amber codon, and thereby the amber codon was suppressed by aminoacyl-$tRNA_{CUA}$ more effectively (Wang et al., 2007). This notable work developing Ribo-X has circumvented one of the intrinsic restrictions that originates from the competition with RF1 on the amber codon.

To overcome the remaining limitation described above, it is required to evolve a new mutant ribosome that is able to use another suppression codon for the incorporation of the second artificial amino acid. Fortunately, such a codon, the so-called four-base codon, inducing +1 frameshift to incorporate an artificial amino acids was already available (Hohsaka et al., 1996); it was even demonstrated that two artificial amino acids were incorporated into the desired specific sites in the combination of amber codon and a four-base codon using an *E. coli* in vitro translation system (Murakami et al., 2003). The team of Chin ingeniously generated a library 16S rRNA mutants based on Ribo-X that focuses on the region of the decoding center, and selected those capable of decoding a four-base codon by the use of aminoacyl-tRNA that had the corresponding anticodon (Neumann et al., 2010). One of the ribosome mutants, referred to as Ribo-Q, was able to effectively read a four-base codon, such as AGGA, UAGA, AAGA, or CCCU, and incorporate an artificial amino acid into a nascent polypeptide chain at one of these codons in *E. coli*. Moreover, the double suppression of AGGA and UAG codons with *p*-azido-L-phenylalanine and N6-[(2-propynyloxy)-carbonyl]-L-lysine paired with the available mutants of MjTyrRS and MbPylRS, respectively, was demonstrated. This remarkable work gives us a new opportunity to explore the technical merits of expressing proteins that contain two or possibly more artificial amino acids in vivo.

Although the decoding efficiency of four-base codon by Ribo-Q was significantly increased compared with that by the WT ribosome, it suffered from the production of a truncated protein as a by-product, due to a failure of the desired frameshift (as shown in Figure S6, S7, and S13 in Neumann et al., 2010). Why? The concrete mechanistic reason for this must wait for the structural analysis of the individual complexes of Ribo-Q with four-base anticodon tRNAs, but the recent X-ray analysis of WT ribosome complexed with some four-base anticodon tRNAs at A site has suggested that the anticodon unlikely forms simple four base pairs with the four-base codon on mRNA (Dunham et al., 2007). When the four-base anticodon tRNA binds to the A site of ribosome, the first base (at position 33.5) in the four-base anticodon possibly unpaired with the forth base in

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Figure 1. Decoding an mRNA Four-Base Codon in a Ribosome
(A) Orthogonal relationship between wild-type ribosome-mRNA and orthogonal ribosome-mRNA.
(B) Decoding four-base codon in the A site.
(C) Decoding four-base codon in the P site. There are multiple possibilities of the decoding mode depending upon the sequences of four-base codon and neighboring codons. Numbers colored in red or black represent numbering of anticodon or mRNA bases, respectively. An expanded base on a quadruplet anticodon is indicated as 33.5 in dark red.

the four-base codon (Figure 1B); alternatively, the second base (at position 34) in the four-base anticodon may sit in the flanking region between the third and forth bases in the four-base codon or even pair with the forth base (Figure 1B). When the four-base anticodon tRNA is translocated to the P site of ribosome after peptidyl-transfer, the frameshift may occur via several possible ways under similar pairing modes albeit with no structural evidence at this time (Figure 1C). This complexity may be further influenced by the neighboring codon (position 5–7) that must be decoded in the designated frame (i.e., after the programmed frameshift). The first three nucleotides of four-base codons are designed by the use of rare codons, such as AGG and CCC, in *E. coli* in order to be efficiently suppressed by the four-base anticodon tRNA rather than naturally occurring tRNAs with three-base anticodon. Thus, the choice of such rare codons limits the creation of four-base codons along with satisfactory suppression efficiencies.

Some challenges still remain for the future exploration to achieve an ultimate goal of protein expression using more than two artificial amino acids in *E. coli*. However, there is no doubt that the successful evolution of Ribo-Q using the orthogonal ribosome-mRNA strategy is a big step toward developing a technology that expresses proteins with multiple artificial amino acids, leading to a new generation of biotechnology.

REFERENCES

Cornish, V.W., Mendel, D., and Schultz, P.G. (1995). Angew. Chem. Int. Ed. Engl. *34*, 621–633.

Dunham, C.M., Selmer, M., Phelps, S.S., Kelley, A.C., Suzuki, T., Joseph, S., and Ramakrishnan, V. (2007). RNA *13*, 817–823.

Hohsaka, T., Ashizuka, Y., Murakami, H., and Sisido, M. (1996). J. Am. Chem. Soc. *118*, 9778–9779.

Murakami, H., Kourouklis, D., and Suga, H. (2003). Chem. Biol. *10*, 1077–1084.

Neumann, H., Wang, K., Davis, L., Garcia-Alai, M., and Chin, J.W. (2010). Nature *464*, 441–444.

Rackham, O., and Chin, J.W. (2005). Nat. Chem. Biol. *1*, 159–166.

Wang, K., Neumann, H., Peak-Chew, S.Y., and Chin, J.W. (2007). Nat. Biotechnol. *25*, 770–777.

Xie, J., and Schultz, P.G. (2006). Nat. Rev. Mol. Cell Biol. *7*, 775–782.

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Original article

Selective estrogen receptor modulators inhibit hepatitis C virus infection at multiple steps of the virus life cycle

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Abstract

We screened for hepatitis C virus (HCV) inhibitors using the JFH-1 viral culture system and found that selective estrogen receptor modulators (SERMs), such as tamoxifen, clomifene, raloxifene, and other estrogen receptor α (ERα) antagonists, inhibited HCV infection. Treatment with SERMs for the first 2 h and treatment 2–24 h after viral inoculation reduced the production of HCV RNA. Treating persistently JFH-1 infected cells with SERMs resulted in a preferential inhibition of extracellular HCV RNA compared to intracellular HCV RNA. When we treated two subgenomic replicon cells, which harbor HCV genome genotype 2a (JFH-1) or genotype 1b, SERMs reduced HCV genome copies and viral protein NS5A. SERMs inhibited the entry of HCV pseudo-particle (HCVpp) genotypes 1a, 1b, 2a, 2b and 4 but did not inhibit vesicular stomatitis virus (VSV) entry. Further experiment using HCVpp indicated that tamoxifen affected both viral binding to cell and post-binding events including endocytosis. Taken together, SERMs seemed to target multiple steps of HCV viral life cycle: attachment, entry, replication, and post replication events. SERMs may be potential candidates for the treatment of HCV infection.
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Keywords: HCV; Tamoxifen; SERM (Selective estrogen receptor modulator)

1. Introduction

Over 170 million people in the world are infected with the hepatitis C virus (HCV). Approximately 20% of infected patients develop cirrhosis and hepatocellular carcinoma after chronic HCV infection. No HCV vaccine is available yet, and the current standard of care, which consists of a combination of interferon (IFN) and ribavirin, is only effective for approximately 50% of infected patients, and many patients have serious side effects. Because of the urgent need for novel HCV therapeutics, research is being conducted to develop new anti-HCV drugs. In addition to *in vitro* screening assays that target HCV-specific enzymes, other approaches that use replicon cells and the recently described Huh 7.5.1-JFH-1 (genotype 2a)-infection system have been developed [1]. The Huh 7.5.1-JFH-1-infection system is an excellent system to identify HCV inhibitors that interfere with individual steps of the HCV life cycle, such as viral attachment, entry, and release. This experimental system allows both viral and host components that are involved in HCV infection to be targeted. Although drugs that target the host components may be toxic, such drugs are unlikely to select for resistant viruses.

We screened chemicals using a cell-based screening system [2] and found that tamoxifen and other selective estrogen receptor modulators (SERMs) inhibited HCV infection. Tamoxifen has been successfully used for the treatment of breast cancer since it was found to be an ER antagonist over 30 years ago. Clomifene and raloxifene, which are compounds

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that are related to tamoxifen, have been developed and used for the treatment of breast cancer and for the treatment of anovulation and osteoporosis. Currently, these three SERMs and toremifene have been approved in Japan and the US, and next-generation SERMs are undergoing clinical evaluation.

Because tamoxifen exhibited the ability to inhibit HCV infection, we determined which SERMs could effectively inhibit HCV infection and be approved for clinical use. The first-generation SERMs—tamoxifen, clomifene, and raloxifene—were all effective against HCV as were other ERα antagonists. We examined whether SERMs could be utilized as new drugs for the treatment of HCV.

2. Materials and methods

2.1. Cells and virus

Human hepatoma cell line, Huh 7.5.1 cells and human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich Co. St. Louis, MO, USA) with 10% fetal bovine serum (FBS). HCV-JFH-1 (HCVcc) (genotype 2a) was the culture supernatant of infected Huh 7.5.1 cells as described previously [2]. A subgenomic replicon cell line, clone #4-1, which harbors the genotype 2a (JFH-1) [3,4], and clone #5-15, that harbors the genotype 1b HCV genome [5], were also cultured in DMEM with FBS.

2.2. Chemicals

The SCADS inhibitor kit I was provided by the Screening Committee of Anticancer Drugs, supported by a Grant-in-Aid for Scientific Research on the Priority Area "Cancer" from The Ministry of Education, Culture, Sports, Science and Technology of Japan. Tamoxifen, diethylstilbestrol, triphenylethylene, 17β-estradiol, and brefeldin A were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Clomifene was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA), and hydroxytamoxifen ((*z*)-4-hydroxytamoxifen) and raloxifene were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Chloroquine was purchased from WAKO (Osaka, Japan). Other chemicals were purchased from Tocris Bioscience (Bristol, UK).

2.3. Quantification of the viral titer in medium

Huh 7.5.1 cells were seeded in 96-well plates at a density of 2×10^4 cells per well in a volume of 120 μl. The next day, 15 μl of media that contained the test compound and 15 μl of the HCVcc virus stock solution at a moi of 0.01 were added to each well. After 5 days, 100 μl of the culture supernatant was taken from each well, and viral RNA was extracted. Total RNA was also extracted from the cells. Quantitative real-time RT-PCR was then performed with One step SYBR PrimeScript RT-PCR Kit (Takara-Bio Co., Otsu, Japan) as described previously [2]. In the case of #4-1 replicon cell, as an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured with primers 5′-CCACCCATGGCAAATTCC-3′ and 5′-TGGGATTTCCATTGAT-3′. Cell growth was monitored using the MTT assay as described previously [6].

2.4. Western blotting

Western blotting was performed as previously described [2]. Briefly, cell lysates that contained equal quantities of protein were separated by SDS-PAGE, transferred onto PVDF membranes, and probed with antibodies against the core antigen (2H9), NS5A (Austral Biologicals, San Raman, CA, USA), or GAPDH (Santa Cruz Biotech. Inc., Santa Cruz, USA). After incubation with horseradish peroxidase-conjugated secondary antibodies, the protein bands on the PVDF membranes were detected using an ECL system (GE Healthcare UK Ltd., Amersham Place, UK).

2.5. Production of and infection with pseudo-particles

HCV pseudo-particles (HCVpp) were generated using the following 3 plasmids: a Gag-Pol packaging construct (Gag-Pol 5349), a transfer vector construct (Luc 126), and a glycoprotein-expressing construct (HCV E1E2) (JFH-1, 2a). The generation of the pseudo-particles was performed according to the method described by Bartosch et al. [7]. To express the glycoproteins of other HCV genotypes, HCV E1E2 constructs of the genotypes 1a (H77), 1b (UKN1B 12.6), 2b (UKN2B 2.8), and 4 (UKN4 11.1) were generously provided by Dr. F. Cosset (INSERM, France) [8]. To produce VSVpp, a plasmid that coded the vesicular stomatitis virus (VSV) envelope, pCAG-VSV, was generously provided by Dr. Y. Matsuura (Osaka University, Japan). Gag-Pol 5349 (3.1 μg), Luc 126 (3.1 μg), and each of the individual glycoprotein-expression constructs (1.0 μg) were co-transfected into 293T cells that were seeded on a 10-cm dish (2.5×10^6 cells) using TransIT-LT1 Transfection Reagent (21.6 μl) (Mirus Bio LLC, Madison, WI, USA). The medium from the transfected cell cultures was harvested and used as the pseudo-particle stock. For the infection assay, Huh 7.5.1 cells were seeded onto a 48-well plate at a density of 4×10^4 cells per well one day prior to infection. The medium was then removed, and the cells were subsequently infected with the pseudo-particles in the presence or absence of drug. The cells were then incubated for 3 h. The VSVpp preparation was diluted (1:600) to infect with similar RLU activity compared to the HCVpp. The supernatant was then removed, fresh culture medium was added to the cells, and the cells were incubated for an additional 3 days. The luciferase assays were performed using a luciferase assay system (Promega Co. Madison WI, USA). Anti-CD81 antibody (sc-23962) was purchased from Santa Cruz Biotech.

3. Results

3.1. Tamoxifen and estrogen receptor α antagonists inhibited HCV infection

Using quantitative RT-PCR, we screened the compounds in the SCADS inhibitor kit I. Drugs and HCVcc at a moi of 0.01

were added to Huh 7.5.1 cells. Five days later, the quantity of HCV RNA in the culture supernatant was measured using quantitative real-time RT-PCR [2]. We found that tamoxifen reduced the levels of JFH-1 RNA in the culture supernatant. We also examined the effects of other SERMs and agonists and antagonists of ERα. As shown in Fig. 1, tamoxifen, clomifene, and hydroxytamoxifen, which have a triphenylethylene backbone, exhibited intense inhibitory effects (EC_{50}: approximately 0.1 μM). Triphenylethylene showed reduced inhibitory activity (data not shown). Raloxifene also inhibited viral RNA production at a similar concentration. (EC_{50}: approximately 0.1 μM) (Fig. 1a). Tamoxifen and raloxifene display both ERα antagonist and agonist properties in a dose- and tissue-dependent manner [9]. In contrast, ICI 182,780 (fulvestrant), ZK164015, and MPP (methyl-piperidino-pyrazole) are exclusively antagonistic [10–12]. These ERα antagonists also showed inhibitory activity against JFH-1, but their EC_{50} values were approximately 1 μM (Fig. 1b). As the 50% toxic concentrations (TC_{50}) for these compounds were observed to be greater than 10 μM (Fig. 1a and b), these specific indexes are over 100. In contrast, the ERα agonists 17β-estradiol, diethylstilbestrol, and PPT (1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrozole) did not inhibit HCV (Fig. 1c). As expected, the SERMs that were observed to effectively inhibit HCV RNA production also reduced the core protein levels intracellularly (Fig. 1d).

3.2. SERMs inhibited more than one step of the JFH-1 life cycle

To determine which step of the JFH-1 life cycle was inhibited by the SERMs studied, we performed time-of-addition experiments. As described previously [2], JFH-1 appears to complete one infectious life cycle in approximately 48 h. Huh 7.5.1 cells were inoculated with JFH-1-containing medium (moi 0.1) with or without drug and were then incubated for 2 h. After the medium was removed, fresh medium with or without drug was added. The cells were then incubated for another 46 h. Treatment with 10 μM tamoxifen for 48 h reduced the amount of viral RNA in the medium to 1.7% of levels observed in the control. Treatment with tamoxifen for the first 2 h after infection (0–2 h) reduced viral RNA to 2.3% of the levels observed in the control. The addition of tamoxifen to the fresh medium just after the removal of the virus (2–48 h) resulted in a reduction in the amount of viral RNA to 10.7% of the levels observed in the control. The addition of tamoxifen 24 h after viral inoculation (24–48 h) resulted in a decrease in the amount of viral RNA to 60% of the levels observed in the control (Fig. 2a). This result suggests that tamoxifen inhibits mainly viral entry and some steps during replication. 10 μM of raloxifene exhibited a similar inhibitory pattern but less inhibited by the treatment after the entry step (Fig. 2b). A pure ERα antagonist, ICI 182,780 (30 μM), also exhibited inhibition of both viral entry and the replication steps, but the inhibition of the entry step was not so marked (Fig. 2c).

To further investigate effect on HCV post replication, we infected HCV in the presence of the drugs for 72 h (moi 0.1) and examined their effects on intracellular and extracellular HCV RNA levels. Brefeldin A, an inhibitor of protein transport [13], was used as a positive control of post replication inhibition. In this experimental setting, brefeldin A showed intracellular HCV RNA accumulation suggesting post replication inhibition (Fig. 2d). SERMs generally reduced HCV RNA in cell as well as HCV RNA in medium, although the extent of reduction was different (Fig. 2d). Lower concentration of SERMs reduced extracellular HCV RNA more robustly than intracellular HCV RNA. At a concentration of 0.1 μM, tamoxifen exclusively inhibited HCV RNA in the culture supernatant but not intracellular HCV RNA levels, in a manner similar to that of brefeldin A (Fig. 2d). The results suggest that SERMs inhibit post replication step(s) such as assembly or release. Because low concentrations of tamoxifen failed to inhibit intracellular HCV RNA, SERMs potentially target post replication step(s) more efficiently than replication step. In this condition, higher concentrations (1 and 3 μM) of tamoxifen seemed to inhibit intracellular HCV RNA rather than extracellular HCV RNA, although the reason is not clear.

To determine the effect of these drugs on chronic infection, we used pre-infected Huh 7.5.1 cells. We infected the cell with HCVcc at a moi of 0.01 and incubated for 3 days. Three days after infection, the drugs were added, and the cells were further incubated for 48 h. At the time of drug addition, the cells were persistently infected, and HCVcc was continuously produced and released into the culture supernatant, which is similar condition to chronic infection. HCV RNA was extracted from the culture supernatant and the cells after 48 h and measured copy number of HCV RNA. Both HCV RNA in the culture supernatant and that in the cell were reduced by treatment with the SERMs, but the intracellular HCV RNA levels were less reduced (Fig. 2e). This suggested that the SERMs caused preferential reduction in extracellular HCV RNA through interference with some post replication step(s), such as assembly or release. Brefeldin A accumulated intracellular HCV RNA, and reduced HCV RNA level in the culture supernatant (Fig. 2e).

These data suggested that the SERMs inhibit multiple steps in the HCV life cycle: entry, viral RNA replication and some post replication step(s).

3.3. SERMs inhibited copies and NS5A protein expression in replicon cells

To confirm the effect of these drugs on viral replication, we used two subgenomic replicon cells. The subgenomic replicon cells, derived from Huh7 cells, harbor HCV viral RNA that replicates autonomously, and they express viral proteins. We treated cells that harbored a subgenomic replicon (#4-1, genotype 2a) [3,4] with the SERMs for 48 h and measured the amount of cellular replicon RNA by quantitative RT-PCR. Treatment with 10 μM of tamoxifen, raloxifene, or 3 μM of clomifene, inhibited HCV RNA compare to GAPDH RNA, although statistical significance was shown in only the inhibition of 10 μM of tamoxifen. ICI 182,780 did not show specific inhibition of HCV RNA (Fig. 3a).

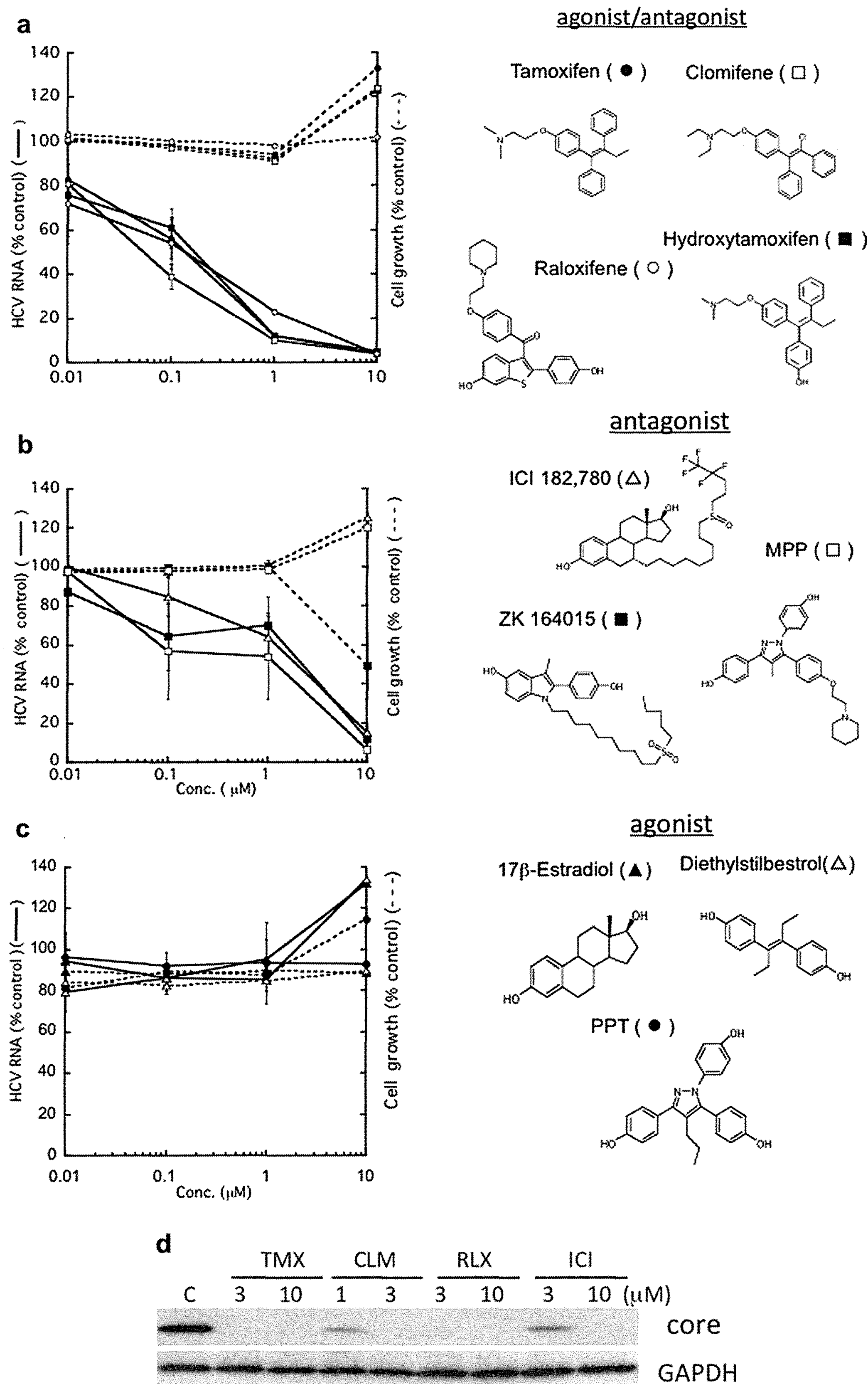

Fig. 1. Effects of SERMs on JFH-1 HCV RNA levels. a) Effects of tamoxifen, clomifene, and raloxifene. Huh 7.5.1 cells were infected with HCV JFH-1 (moi 0.01) in the presence of drugs and were incubated for 5 days. Drugs were added just before viral inoculation. HCV RNA in the medium was measured by tube-capture-RT-PCR [2]. Parallel cultures of cells without virus were analyzed using the MTT assay to detect the inhibition of cell growth due to drug exposure. Tamoxifen (closed circles), clomifene (open rectangles), hydroxytamoxifen (closed rectangles), and raloxifene (open circles). The percentages to control HCV RNA and

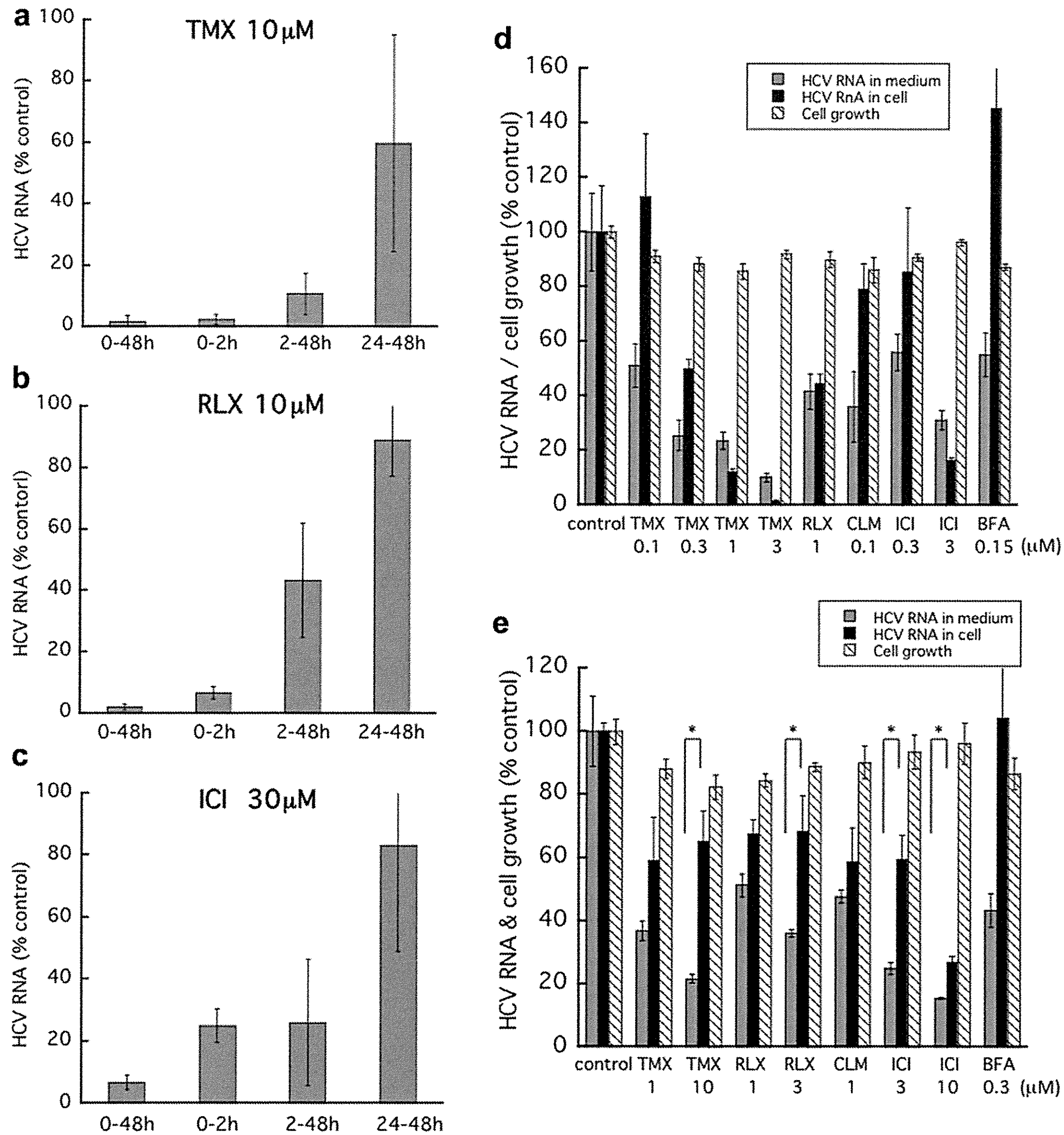

Fig. 2. Time-of-addition experiments (a–c) and the effect of SERMs on extracellular and intracellular HCV RNA in simultaneous infection (d) and in persistent infection (e). Huh 7.5.1 cells were treated with tamoxifen (TMX) (10 μM, a), raloxifene (RLX) (10 μM, b), or ICI 182,780 (ICI) (30 μM, c) during the following time periods: 0–2 h, 2–48 h, or 24–48 h after JFH-1 infection (moi 0.1). Forty-eight hours after infection, the culture supernatant was harvested, and HCV RNA was extracted and subjected to quantitative real-time RT-PCR to determine the number of copies of the JFH-1 genome. The data are the averages of three independent experiments and the standard deviation. d) Effect of treatment with SERMs for 3 days on extra- and intra-cellular HCV RNA levels. Huh 7.5.1 cells were infected with JFH-1 (moi 0.1) just after addition of the SERMs. Three days later, RNA was extracted from the cells and from the culture supernatant. The amount of HCV RNA was measured by quantitative real-time RT-PCR. Brefeldin A (BFA) was used as a positive control. e) Huh 7.5.1 cells were infected at a moi of 0.01, 3 days before addition of drugs. The infected cells were treated with SERMs for 48 h. RNA was subsequently extracted from the cells and the culture supernatant to determine the viral genome copy number. The results are presented as the percentage of control cells without drug. The data are the averages of triplicates and the error bars represent standard deviation. *P-value < 0.05. One representative experiment of two independent experiments is shown.

Next we treated this (#4-1, genotype 2a) and another replicon (#5-15, genotype 1b) [5] with the SERMs for 3 days and examined the effect of the compounds on the HCV NS5A protein levels by western blotting. As shown in Fig. 3b, the SERMs except ICI 182,780 reduced the level of NS5A in accordance with the results in Fig. 3a. ICI 182,780 seemed to slightly reduce NS5A protein in #5-15 replicon cell. The SERMs did not reduce the protein levels of GAPDH in the subgenomic replicon cells (Fig. 3b). These results indicated that SERMs, at least tamoxifen, raloxifene and clomifene,

control cell growth are indicated by solid lines and dotted lines, respectively. b) Effect of the following ERα antagonists: ICI 182,780 (closed triangles), ZK164015 (closed rectangles), and MPP (open rectangles). c) Effect of the following ERα agonists: 17β-estradiol (closed triangles), diethylstilbestrol (open rectangles), and PPT (closed circles). The results are presented as percentages of the control cells that were not treated with drugs. Values are the averages of triplicates, and the error bars represent the standard deviation of the mean. One representative experiment of three independent experiments is shown. d) Huh 7.5.1 cells were infected (moi 0.01) in the presence of tamoxifen (TMX), clomifene (CLM), raloxifene (RLX), or ICI 182,780 (ICI) and incubated for 5 days. Cell lysates were blotted with anti-core and anti-GAPDH antibodies as described in the Section Materials and methods.

were effective not only against HCV genotype 2a but also HCV genotype 1b and that the compounds inhibited a HCV replication step. The growth of the replicon cells was suppressed by treatment with 10 μM of clomifene. Clomifene at concentrations less than 10 μM and tamoxifen, raloxifene and ICI 182,780 at 10 μM concentration or lower did not inhibit cell growth (Fig. 3c).

3.4. SERMs inhibited entry of HCVpp but not VSVpp

To further examine the inhibition of early viral processes by the SERMs, we used infectious HCV pseudo-particles (HCVpp). Because HCVpp enter into cell dependent on HCV envelope protein but replicate dependent on retroviral system in the cell, we can exclude other effects of the drug except effect on HCV entry system. Pseudo-particles with the viral envelope glycoprotein mimic the entry of the parental virus, and this system has been used for investigation of HCV entry [7,8,18,20,21]. The infectious titer is determined by luciferase activity. We added tamoxifen to HCVpp- or VSVpp-containing medium and incubated Huh 7.5.1 cells with this medium for 3 h. After washing the cells, fresh medium was added, and the cells were incubated for 3 days. Treatment with tamoxifen reduced the luciferase activity of the cells that were infected with HCVpp in a dose-dependent fashion. In contrast, the luciferase activity caused by VSVpp was not reduced by the same concentrations of tamoxifen (Fig. 4a). We also examined the effect of other SERMs, such as clomifene, raloxifene, ICI 182,780, ZK164015, and MPP, on HCVpp infection. All of these SERMs inhibited the luciferase activity caused by HCVpp but not the activity caused by VSVpp (Fig. 4b). ICI 182,780 showed a weaker effect compared to tamoxifen, clomifene and raloxifene. Next, we examined the effects of these drugs on various genotypes of HCVpp. Although the extent of inhibition was varied, the compounds inhibited all of the genotypes that were examined (Fig. 4c). At a concentration of 10 μM, ICI 182,780 inhibited all of the genotypes of HCVpp other than genotype 2a. These results suggested that the SERMs inhibit entry of all genotypes of HCV.

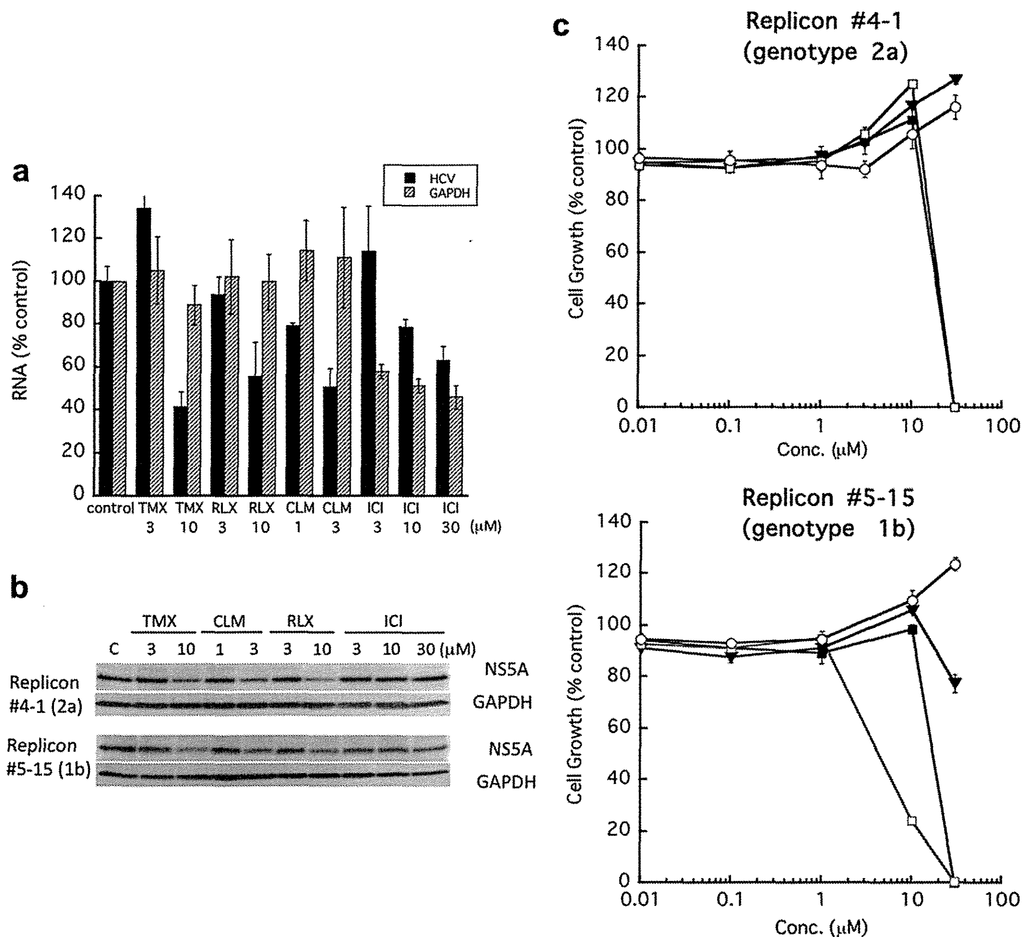

Fig. 3. The effect of SERMs on cells that harbored a subgenomic replicon. A subgenomic-replicon-harboring cell line clone #4-1 (genotype 2a) was treated with SERMs for 48 h. The total RNA was extracted from the cells, and amount of HCV RNA genome was measured. As an internal control, relative amount of GAPDH RNA was measured and indicated as percentage of control cells without drug (a). Another subgenomic-replicon-harboring cell line, clone #5-15 (genotype 1b) was treated with SERMs for 3 days. Cell lysates were subjected to western blotting with an anti-NS5A antibody or an anti-GAPDH antibody (b). Cells that were grown for 3 days in the presence of tamoxifen (closed rectangles), clomifene (open rectangles), raloxifene (closed triangles), or ICI 182,780 (open circles) were measured using the MTT assay. Cell growth is expressed as a percentage of control cells without drug (c). The values are the average of triplicate and the error bars represent the standard deviation of the mean. One representative experiment of two independent experiments is shown.

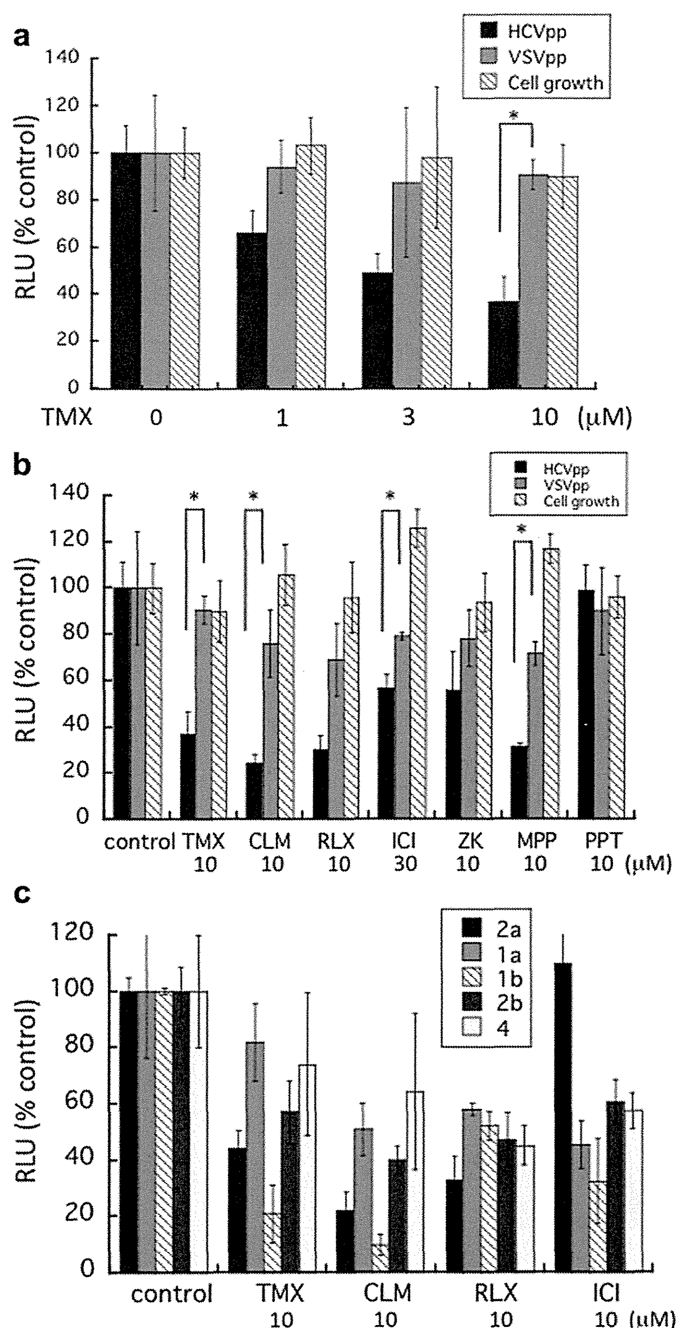

Fig. 4. Effect of SERMs on HCV pseudo-particle (HCVpp) infection. a) Huh 7.5.1 cells were incubated with pseudo-particles (HCVpp or VSVpp) in the presence or absence of tamoxifen for 3 h. The supernatants were removed, medium was added back to the cell cultures, and the cells were incubated for another 3 days. The VSVpp preparation was diluted 600 times so it was infected at similar RLU activity levels compared to HCVpp (approximately 5000 RLU). A parallel culture without pseudo-particles was analyzed using the MTT assay to evaluate the effect of the drugs on cell growth. b) Effects of various SERMs on HCVpp and VSVpp infection. c) Effects of SERMs on the various genotypes of HCVpp infection. The control luciferase activities were approximately 5000 RLU (genotype 2a), 3000 RLU (genotype 1a), 2400 RLU (genotype 1b), 3900 RLU (genotype 2b), and 860 RLU (genotype 4). The values are expressed as the percentage of control cells without drug. The data are the averages of three wells and the error bars are the standard deviation of the mean. *P-value < 0.05. One representative experiment of three independent experiments is shown.

3.5. Effect of tamoxifen on the attachment and entry steps

To better understand how tamoxifen blocks HCV entry, we performed an experiment to discriminate between the inhibition of HCV attachment to cells and the inhibition of post-binding entry events. HCV attaches to several cellular receptors via its E1 and E2 envelope proteins and enters via clathrin-mediated endocytosis [14–16]. We used HCVpp because infection with HCVpp is thought to simulate HCV entry [7,17,18] and the entry is independent of HCV replication. HCVpp binding to the cellular receptors was performed at 4 °C for 1.5 h. Under these conditions, HCVpp bind to the cells but entry is not efficient. The inoculum was removed, and fresh medium was added to the cells. The cells were subsequently incubated at 37 °C. In protocol I, the drug was administered during the binding step at 4 °C. After the shift to 37 °C, treatment with the drug was performed during first hour (protocol II) or after 1 h at 37 °C (protocol III) to distinguish between the inhibition of early and late post-binding events (Fig. 5a). The inoculum was removed after treatment, and fresh medium was added to the cells. We used chloroquine, a lysosome-tropic agent, as a control inhibitor for early entry (protocol II) [19]. We also used an anti-CD81 antibody that specifically inhibits HCV entry through the inhibition of the HCV cellular receptor protein CD81 at early entry [20,21]. As expected, chloroquine inhibited luciferase activity when the cells were treated during the early post-binding step (protocol II). This result suggested that endocytosis occurred primarily during the first post-binding period (protocol II). Anti-CD81 markedly inhibited luciferase activity during protocol II as reported [18,19]. Tamoxifen treatment did not result in clear differences between the protocols and the compound displayed similar activity regardless of the treatment period (Fig. 5b left). As a control, the same experiment was performed using VSVpp. Chloroquine inhibited the early entry step of VSVpp, but anti-CD81 and tamoxifen did not show any inhibition (Fig. 5b right).

Tamoxifen is a lipophilic weak base and inhibits acidification intracellularly [22]. Therefore, we examined whether the inhibition of the endocytosis of HCVpp by tamoxifen was dependent on its function as a weak base. Chloroquine is a weak base and inhibits endosome acidification. The pH sensitivity is considered a good indication of clathrin-dependent endocytosis. Previous reports have indicated that chloroquine inhibited HCVcc and HCVpp infection [14,19]. We adjusted the medium to pH 5.5 and incubated the cells in this acidic medium in the presence or absence of tamoxifen for 2 h post-binding. The acidification of the medium did not affect either the entry of HCVpp or the cell growth (Fig. 5c). Treatment with tamoxifen in the medium with a normal pH (pH 7.1) reduced HCVpp entry, and treatment with the drug in the acidic medium also reduced entry to a similar extent. In contrast, chloroquine treatment in regular medium reduced HCVpp entry, but entry was restored in the acidic medium (Fig. 5c). These results indicate that the inhibitory effect of tamoxifen was not dependent on the function of this compound as a base, unlike the effects of chloroquine.