Application of the Yeast-Surface-Display System for Orally Administered Salmon Calcitonin and Safety Assessment

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High manufacturing costs and oral delivery are the constraints in clinical application of calcitonin. We selected surface-displayed Saccharomyces cerevisiae *as a low-cost and safe carrier for oral delivery of salmon calcitonin (sCT). The sCT DNA fragment, optimized according to the codon preference of* S. cerevisiae*, was synthesized and cloned into the plasmid M-pYD1 to yield recombinant yAGA2-sCT, which was induced to express sCT by galactose for 0, 12, and 24 h. sCT expression was detected on the cell surface by indirect immunofluorescence and peaked at 12 h. About 65% recombinants expressed sCT on flow cytometry. The in vivo and in vitro activity of recombinant sCT was determined by detecting bioactivity of antiosteoclastic absorption on bone wafers and orally administering yAGA2-sCT to Wistar rats, respectively. For safety assessment of yAGA2-sCT, we observed abnormalities, morbidity, and mortality and determined body weight, serum chemistry parameters, hematological parameters, and organ weight. In vitro bioactivity of the recombinant sCT was similar to that of commercial sCT, Miacalcic; oral administration of 5 g/kg yAGA2-sCT induced a long-term hypocalcemic effect in Wistar rats and no adverse effects. This study demonstrates that yAGA2-sCT anchoring sCT protein on a* S. cerevisiae *surface has potential for low-cost and safe oral delivery of sCT.* © 2010 American Institute of Chemical Engineers *Biotechnol. Prog.*, 26: 968–974, 2010
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Introduction

Oral administration is the most convenient and comfortable way of drug delivery method. An increasing number of drugs are protein and peptide based. At present, protein-based drugs are usually administered by injection, whereas the per-oral route is still the most intensively investigated for the advantages of ease of dosing administration, patient compliance, and flexibility in formulation. New strategies, drug delivery systems (DDSs), have been introduced to control the pharmacokinetics, pharmacodynamics, nonspecific toxicity, immunogenicity, biorecognition, and efficacy of drugs. DDSs have been developed to deliver drugs such as genes and proteins at a controlled rate or by target. Nanoparticles and nanoformulations have been applied as DDSs with great success for gene therapy, antitumour therapy, and protein delivery.[1-6] However, researchers are still finding new ways to administer parenteral medications orally and safely and to lower the cost of production, delivery, and long-term therapy.

Calcitonin (CT) is a kind of peptide hormone consisting of 32 amino acids, with an N-terminal disulfide bridge and a C-terminal prolineamide residue. It plays crucial physiological roles in calcium homeostasis and bone remodeling.[7,8] A variety of CTs were discovered from natural organisms and named human calcitonin (hCT), eel calcitonin (eCT), and salmon calcitonin (sCT); for example, sCT is widely applied in the clinic because of its high potency and analgesic properties. sCT is mainly used to treat metabolic bone disease, such as osteoporosis and Paget's disease, hypercalcemia, osteoarthritis, and bone-associated pain conditions.[9] Currently, sCT is limited to parenteral or nasal administration.[10] To effectively inhibit the phenomena of metabolic bone disorders, such as osteoporosis and Paget's disease, a frequent and relatively high dosage of CT is administered,[11] which leads to poor patient compliance during chronic treatment and limitations in CT utility. Oral delivery is therefore the preferred route of administration for peptide and protein drugs, such as sCT, for long-term treatment. However, without considering production costs, a major obstacle of oral delivery of small peptides is the high acid content and vast array of peptidases and other enzymes in the digestive tract, which degrades most peptides before their absorption into the bloodstream.[12]

To administer CT orally, various chemical materials have been attempted as carriers, such as polystyrene nanoparticles,[13] double liposomes,[14] pH-sensitive microsphere,[15]

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lipid nanoparticles,[16] and chitosan–pentaglycine–phenylboronic acid conjugate.[17] All these chemical materials were loaded with sCT (or hCT) by complex processes and at high cost, which hinders the application of sCT. Recently, genetically modified biomaterial appears to be a promising alternative, a new concept of "biodrugs."[18] Furthermore, an intracellular-expressed heterologous protein was successfully delivered orally to an artificial gastrointestinal system with recombinant *Saccharomyces cerevisiae* used as a "biodrug."[19] *S. cerevisiae* shows high resistance to enzymes in the digestive tract[19] and has an extensive history of safe use in the food industry, which allows for its wide application. Currently, a *S. cerevisiae* surface-display system has been used for genetically anchoring enzymes, functional proteins, and antibodies on the cell surface.[20]

The aim of the current study is to explore a new strategy for oral delivery of the peptide drug sCT by the surface-display *S. cerevisiae* system. We also assayed the in vitro and in vivo bioactivity and safety of the recombinant sCT.

Materials and Methods

Materials

The pYD1 yeast Display Vector kit and antibodies were from Invitrogen (Shanghai, China). Restriction enzymes were from Takara (Dalian, China). Miacalcic (sCT injection, 5,000 IU/mg) was from Novartis AG (Switzerland). Male Wistar rats (210 ± 20 g), male Wistar rats (90 ± 10 g), and Wistar rat pups (4- to 6-day-old) were from Medicament Inspection Institution (Tsingdao, China).

Plasmid and strain construction

The sCT sequence was designed according to the biased codon of *S. cerevisiae*, and the single-strand sCT DNA was artificially synthesized. The double-strand sCT DNA was prepared by annealing single-strand DNA SCT1 (5′-CGGGATCCTGTTCTAACTTGTCTACCTGTGTTTTGGGTAAGTTGTCTCAAGAATTGCACAAGTTGCAAACCT-3′) and SCT2 (5′-CGGGATCCTTATCATTAACCTGGAGTACCAGAACCAGTGTTAGTTCTTGGGTAAGTTTGCAACTTGTGAGCGTC-3′) together as previously described.[21] Plasmid M-pYD1 was modified from pYD1 by inserting the *S. cerevisiae URA3* gene to both ends of the expression cassette to integrate the target gene into the chromosome of *S. cerevisiae*. The *Bam*H I-digested and purified sCT DNA was inserted into plasmid M-pYD1 after the enterokinase cut site to yield M-pYD1-sCT. After the M-pYD1-sCT and the empty vector of M-pYD1 were digested with *Nco* I enzyme at the *URA3* site, the DNA fragment containing the expression cassette of sCT (or V5 as a control) was transformed into the *S. cerevisiae* strain EBY100 (*trp1 leu2 Δ1 his3 Δ200 pep4::HIS2 prb Δ1.6R can1 GAL*) to yield yAGA2-sCT (or yAGA2 control) as described.[22]

Protein expression and immunofluorescence analysis

Transformants were grown in SD-CAA (4% dextrose, 0.67% yeast nitrogen base, and 1% casamino acids) at 30°C for <20 h. To induce protein expression, yeast cells were centrifuged, suspended to an absorbance <1 OD_{600} in SG-CAA (2% galactose, 0.67% yeast nitrogen base, and 1% casamino acids), and cultured at 30°C. Cells at 2 OD_{600} induced after 0, 12, and 24 h were collected and placed on ice. About $<10^7$ cells were washed with phosphate buffered saline containing 0.5% bovine serum albumin and incubated for 1 h at 4°C with the primary antibody rabbit-anti-V5 or rabbit-anti-CT antibody to yAGA2 and yAGA2-sCT, respectively. Cells were washed with PBS containing 0.5% BSA and incubated for 1 h at 4°C with goat-anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC). The labeled yeast cells were analyzed on a Coulter Epics XL flow cytometer (Beckman Coulter).

Yeast culture and freeze-drying conditions

Transformants were precultured to stationary phase at 30°C in SD-CAA broth. A 1/10 dilution was transferred into SG-CAA broth and grown in a shaking incubator (30°C, 200 rpm, and 36 h) until the cell density reached 5×10^7 cells/mL. Cells were harvested (4°C, 5000 g, and 10 min) at the beginning of their stationary growth phase,[23] suspended in physiological sterile water, and frozen at −40°C with a cooling rate of 1°C/min.[24] Freeze drying was for 24 h at a condenser plate temperature of −40°C and a chamber pressure of <6 Pa. During the last 2 h of secondary drying, samples were heated at 23°C to minimize residual moisture content.[25]

Preparation of sCT solution for cell experiment

In total, 10-mg lyophilized yAGA2-sCT strain was dissolved in 500 μL buffer containing 50 mM Tris-HCl (pH 5.6) and 5 mM $CaCl_2$, and incubated with 1 U native enterokinase at 21°C for 24 h. The solution was centrifuged for 10 min at 5,000*g*. The obtained supernatants (i.e., sCT solution) were used in the cell experiment. In total, 10-mg lyophilized yAGA2 strain underwent the same procedure to yield CK solution as the negative control.

Osteoclastic resorption on bone wafers

Bovine diaphyseal cortical bone wafers (~4 mm × 4 mm × 0.3 mm) were cut into a substrate by use of a low-speed diamond saw. The wafers were immersed in 70% ethanol, cleaned by ultrasonication for 6 min, and then underwent multiple rinses in sterile water. The osteoclasts were isolated from the long bones (femurs and tibias) of 4- to 6-day-old euthanized rat pups as described previously.[26] The supernatant of osteoclast suspension was aliquoted (100 μL) into the wells of a 96-well plate, each well having one bone wafer as the substrate. Cultures were incubated for 7 days. One day after cultivation, 10-μL Miacalcic (containing 10^{-8} M sCT), 10-μL CK, or 10-μL sCT prepared previously was added into wells, respectively. The entire bone wafer was digitally photographed at 200× magnification. The areas of osteoclast resorption pits were measured by the use of image analysis software (Adobe Photoshop) as described.[27] Six bone wafers were analyzed per treatment.

Hypocalcemic experiment in normal rats

Seven-week-old male Wistar rats (210 ± 20 g) fasted for 12 h but had free access to water before experiments.[28] To investigate the hypocalcemic effect of yAGA2-sCT, rats were randomly divided into six groups ($n = 6$) for treatment: 5 g/kg lyophilized yAGA2 by oral administration; 2 IU/kg Miacalcic by oral administration; lyophilized yAGA2-sCT (0.1, 0.5, and 5 g/kg) by oral administration; or 200 mIU/kg

Miacalcic by subcutaneous injection. The ionized calcium concentration in blood (~0.1 mL) obtained from the tail vein at different times was measured with the use of an analyzer with calcium electrodes. The changes in calcium concentration before and after all treatments were calculated, and means and standard errors (SE) were determined. Each value was plotted as a function of time. We used area under the receiver operating curve (AUC) analysis. The area between the curve for the change in the ionized calcium concentration vs. time and a horizontal line representing zero change was also calculated by the trapezoidal method until the ionized calcium concentration returned to the initial value. The value obtained (area of ionized calcium reduction) was used as an index of the biological effect of sCT, along with a minimum ionized calcium concentration level. Statistical significance was obtained by comparing with the group receiving 5 g/kg lyophilized yAGA2.

Safety assessment

Administration. In total, 40 male Wistar rats (90 ± 10 g) were divided into four groups ($n = 10$ each). Before treatment, all animals were observed for 1 week, and the experiment was started when no abnormality appeared in their activity, diet, or feces. The three test groups underwent oral gavage with 0.1, 0.5, and 5 g/kg yAGA2-sCT (equal to 0.016, 0.08, and 0.8 g/kg, respectively, of clinical medication for humans) daily for 30 consecutive days. The vehicle group underwent oral gavage with water instead of yAGA2-sCT.

Behavior Observation and Body Weights. All rats were observed twice daily for behavior, morbidity, and mortality at the time of treatment and about 1 h after treatment. Individual body weights were recorded weekly until the rats were sacrificed.

Pathology

Hematological parameters (white blood cell count, red blood cell count, platelet counts, hemoglobin, lymphocytes, and neutrophils) were determined by the use of a Sysmex KX-21 analyzer (Sysmex Corporation, Kobe, Japan) according to the operator's manual.

Serum levels of albumin, alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, cholesterol, creatinine, glucose, total protein, and triglycerides were determined by the use of an Olympus, AU400 automatic biochemical analyzer (Olympus, Tokyo, Japan).

All animals underwent complete necropsy. At the time of necropsy, the following tissues and organs were collected and weighed: heart, lung, kidneys, liver, spleen, and testes. Paired organs were weighed together. Ratios of organ to final body weight were calculated.

Statistical analysis

Statistical significance was determined by the Student's t-test. A $P < 0.05$ was considered statistically significant.

Results

Design and cloning of the sCT DNA

The sCT gene was designed to be coded with *S. cerevisiae*-biased codons[29] to obtain an increase in expression level. Figure 1 shows the original DNA sequence of sCT and the optimized one with the same protein product. The sequencing results agreed well with the designed sequence. For plasmid M-pYD1-sCT, the sCT gene with an enterokinase-cut site was fused to the C-terminal portion of Aga2 under the control of GAL1 promoter; a glycin was appended to the 3′ end of the sCT gene as the potential amidation site for peptidylglycinea α-amidation monooxygenase, followed by 3 stop codons. *S. cerevisiae URA3* gene was inserted into both ends of an expression cassette in plasmid M-pYD1-sCT to integrate the sCT gene into *S. cerevisiae* chromosome and to produce successive generation of the recombinants. The α-agglutinin receptor of *S. cerevisiae* was used to display sCT on the cell surface. This receptor consists of two subunits encoded by the *AGA1* and *AGA2* genes. The Aga1 protein (Aga1p, 725 amino acids) is secreted from the cell and becomes covalently attached to glucan in the extracellular matrix of the yeast cell wall. The Aga2 protein (Aga2p, 69 amino acids) binds to Aga1p through two disulfide bonds and, after secretion, remains attached to the cell by its contact with Aga1p.[30]

Natural sCT	TGC	TCC	AAC	CTC	AGC	ACC	TGT	GTG	CTG	GGC	AAA	CTG											
Optimized sCT	TGT	TCT	AAC	CTG	TCT	ACC	TGT	GTT	TTG	GGT	AAG	TTG											
Amino acids	C	S	N	L	S	T	C	V	L	G	K	L											
Natural sCT	TCC	CAA	GAG	CTG	CAC	AAA	TTG	CAG	ACG	TAC	CCC	CGC											
Optimized sCT	TCT	CAA	GAA	TTG	CAC	AAG	TTG	CAA	ACC	TAC	CCA	AGA											
Amino acids	S	Q	E	L	H	K	L	Q	T	Y	P	R											
Natural sCT	ACC	AAT	ACG	GGA	AGT	GGC	ACG	CCT															
Optimized sCT	ACC	AAC	ACC	GGT	TCT	GGT	ACT	CCA	GGT	TAA	TGA	TAA											
Amino acids	T	N	T	G	S	G	T	P	G	*	*	*											

Figure 1. Nucleotide and amino acid sequences of natural salmon calcitonin (sCT) and optimized sCT.

Induced expression of sCT protein

The sCT protein was detected on the yeast yAGA2-sCT by indirect immunofluorescence labeling with rabbit-anti-CT antibody (Figures 2a,b). In contrast, yeast EBY100 cells that did not express sCT (Figures 2c,d) could not bind the rabbit-anti-CT antibody and did not exhibit intense green fluorescence on fluorescence microscopy (Figure 2c).

The surface expression levels of yAGA2-sCT or yAGA2 were estimated by analyzing FITC-labeled sCT protein or V5 epitope tags, respectively. The percentage of expressed cells in 10,000 cells was calculated on flow cytometry. After yAGA2-sCT or yAGA2 was induced by galactose from 0 to 24 h, flow cytometry revealed two peaks at the induction time of 12 h and only one peak at 0 or 24 h. The amount of cells expressing the sCT protein or V5 epitope tags increased with induction time prolonged to 12 h and decreased subsequently. The results agreed with those observed from fluorescence microscopy at 0, 12, or 24 h. The percentage of FITC-labeled yAGA2-sCT reached 65% after 12-h induction and was higher than that of yAGA2 cells (52%) (Figure 3). Therefore, the use of biased codons of *S. cerevisiae* in the sCT gene might facilitate the expression of sCT protein.

Antiosteoclastic bone resorption of recombinant sCT

The recombinant sCT protein was released from the cell surface with the aid of enterokinase digestion and cultured

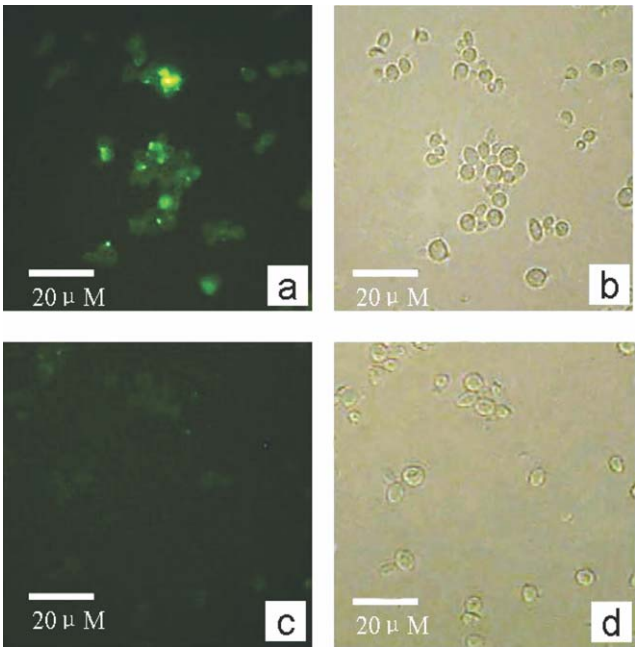

Figure 2. Immunofluorescence labeling of yeast yAGA2-sCT.

(a, b) sCT displayed on the yAGA2-sCT cell surface and (c, d) the host EBY100, which did not express sCT on the cell surface. Labeling was performed with rabbit-anti-calcitonin antibodies and goat-anti-rabbit-FITC. Left panels, immunofluorescence micrographs; right panels, phase-contrast micrographs.

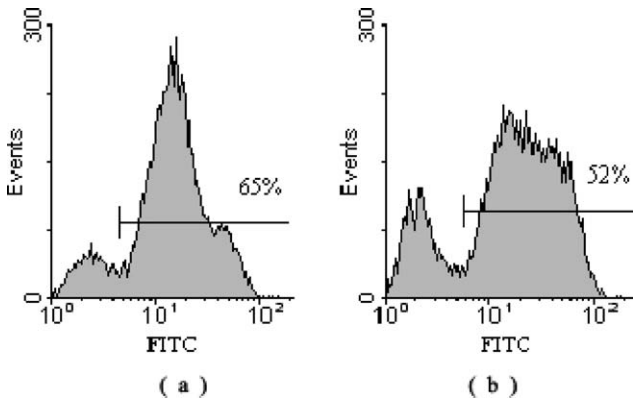

Figure 3. sCT and V5 (positive control) protein expression on yeast surface after the recombinants were induced by galactose for 12 h.

(a) sCT on yAGA2-sCT cell surface was stained with anticalcitonin antibody and goat-anti-rabbit-IgG-FITC. (b) V5 on yAGA2 cell surface was stained with anti-V5 antibody and goat-anti-rabbit-IgG-FITC. 10,000 cells were analyzed by use of a Coulter Epics XL flow cytometer. The percentage of expressed cells is indicated.

with the osteoclasts to investigate the effect of the protein on resorption bioactivity of osteoclasts. sCT bound directly to the CT receptor on osteoclasts, which resulted in inhibited bone resorption and then decreased area of lacuna for osteoclasts in the bone wafer substrate. The mean area of lacuna in the bone wafers treated with Miacalcic (10^{-8} M sCT) or sCT solution was 688.60 ± 34.79 and 696.25 ± 29.04 μm^2, respectively, which was significantly lower than that with CK solution (1381.52 ± 44.97μm^2) ($P < 0.01$) (Figure 4). The results suggested that sCT solution from the yAGA2-sCT significantly inhibited the resorption bioactivity of osteoclasts as well as Miacalcic did (positive control).

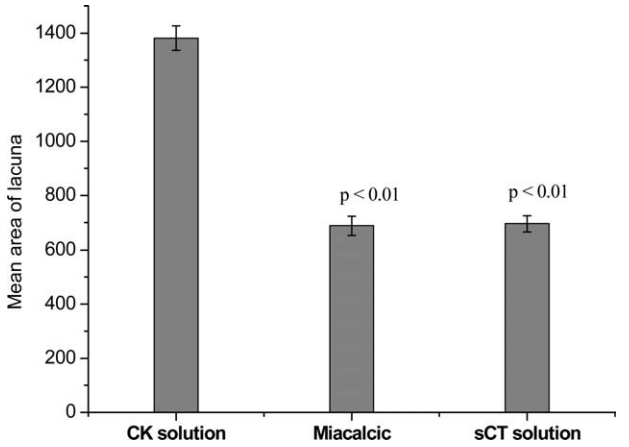

Figure 4. The mean area of lacuna in bone wafers treated with CK solution (negative control), Miacalcic (positive control), and sCT solution for 7 days.

Hypocalcemic effect induced by oral administration of yAGA2-sCT

Hypocalcemic effect was indicated by two parameters: the minimum ionized calcium concentration level of the initial (Ca_{Min}) and the area of ionized calcium reduction (AUC analysis). No significant Ca_{Min} or AUC was observed with Miacalcic (2 IU/kg) or yAGA2-V5 strain (5 g/kg) administration, which suggested that neither could induce the hypocalcemic effect. The Ca_{Min} with 200 mIU/kg Miacalcic injection (85.53% ± 1.2 $P < 0.01$) and oral administration of 5 g/kg yAGA2-sCT (87.71% ± 1.3 $P < 0.5$) significantly differed from yAGA2-V5 treatment (negative control) (Table 1), which suggested that both treatments induced an hypocalcemic effect in Wistar rats. Furthermore, the Ca_{Min} decreased with increasing dose of yAGA2-sCT strain, from 0.1 g/kg (93.8%) to 0.5 g/kg (91.2%) or 5 g/kg (87.7%), and the AUC increased from 0.47 to 1.08 or 1.65 mM h, respectively (Table 1). Although no hypocalcemic effect was observed in the groups treated with 0.1 or 0.5 g/kg yAGA2-sCT, the AUC with 5 g/kg yAGA2-sCT treatment was significantly higher than that with yAGA2-V5 treatment (negative control). Interestingly, the action time was longer for 5 g/kg yAGA2-sCT oral administration than with 200 mIU/kg Miacalcic injection, and the AUC with 5 g/kg yAGA2-sCT (1.65 ± 0.07 mM h) was almost three times higher than that with 200 mIU/kg Miacalcic injection (0.58 ± 0.06 mM h), which indicates that oral administration of 5 g/kg yAGA2-sCT to rats can induce a long-term hypocalcemic effect. This effect was confirmed by the concentration–time profiles of ionized calcium in serum after various treatments in rats, which showed lower change in serum calcium concentration over time with 5 g/kg lyophilized yAGA2-sCT than with other treatments (Figure 5).

Safety assessment of orally administrated yAGA2-sCT

We found no instances of abnormality, morbidity, and mortality during the experiment. The body weight of rats receiving 0.1, 0.5, and 5 g yAGA2-sCT did not differ from that with the vehicle-treated rats (Figure 6). yAGA2-sCT has no obvious body weight effects on the test groups.

We found no treatment-related or adverse effects of yAGA2-sCT on hematology after 30 days of consecutive administration (Table 2). The serum level of hemoglobin, white blood cell count, red blood cell count, and platelet

Table 1. Minimum Calcium (Ca_{Min}) and Area Under the Curve (AUC) Values for Rats Treated with Different Reagents

Group	Reagent	Administration	Dose	Ca_{Min}* (%)	AUC† (mM h)
1	yAGA2-V5	Oral	5 g/kg	95.34 ± 0.9	0.21 ± 0.05
2	Miacalcic	Oral	2 IU/kg	96.16 ± 1.1	0.28 ± 0.05
3	Miacalcic	Injection	200 mIU/kg	85.53 ± 1.2‡	0.58 ± 0.06§
4	yAGA2-sCT	Oral	0.1 g/kg	93.81 ± 1.2	0.47 ± 0.06
5	yAGA2-sCT	Oral	0.5 g/kg	91.18 ± 2.1	1.08 ± 0.21§
6	yAGA2-sCT	Oral	5 g/kg	87.71 ± 1.3§	1.65 ± 0.07‡

* minimum serum ionized calcium concentration level compared with the initial concentration level (mean ± SE). † Area between changed serum ionized calcium concentration vs. time and horizontal at zero change (mean ± SE). ‡ $P < 0.01$ and § $P < 0.05$ compared with group 1.

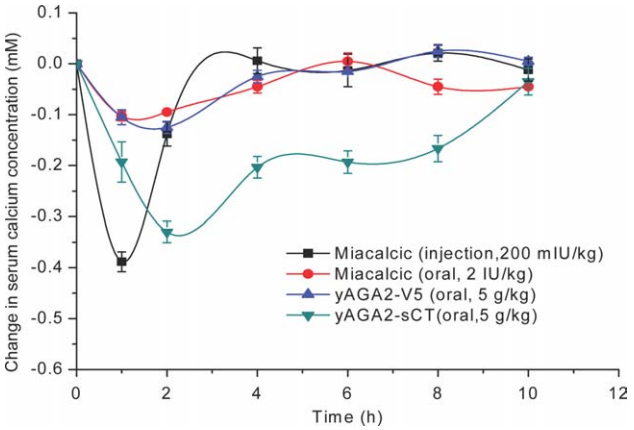

Figure 5. Concentration–time profiles of ionized calcium in serum after oral administration of 5 g/kg lyophilized yAGA2-V5, oral administration of Miacalcic, subcutaneous injection of Miacalcic, and oral administration of 5 g/kg of lyophilized yAGA2-sCT strain in rats.

Each value represents the mean ± SE of six experiments.

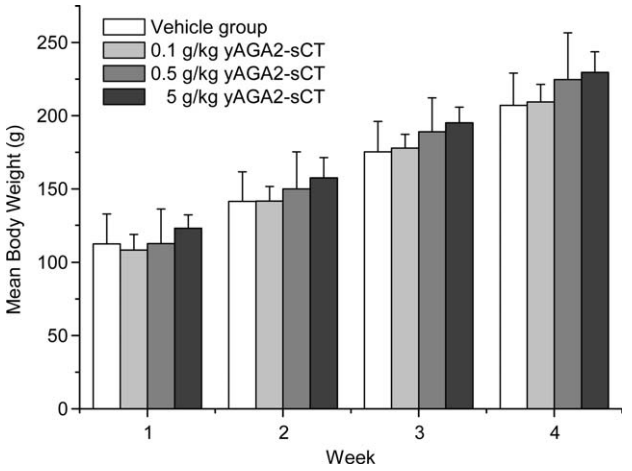

Figure 6. Mean body weight of Wistar rats administered yAGA2-sCT.

counts in the yAGA2-sCT-treated groups did not significantly differ from those in the vehicle-treated group ($P > 0.05$). In all yAGA2-sCT-treated groups, red blood cell count and hemoglobin level were normal and stable, and no anemia appeared; the white blood cell and platelet counts were both stable, which indicated no evidence of infection or inflammatory reaction. Administration of yAGA2-sCT had no obvious effects on the ratio of lymphocytes and neutrophils in white blood cells. Therefore, no infection of virus or bacteria was observed in the test rats.

Similarly, serum levels of albumin, alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, cholesterol, creatinine, glucose, total protein, and triglycerides were not affected by administration of yAGA2-sCT (Table 3). So, the function of liver and kidney in the tested rats was normal, without pathological symptoms. No macroscopic findings were evident on necropsy in all the animals.

The color and organ size for yAGA2-sCT groups were as normal as those for the vehicle-treated group, and we did not observe effusion, hyperplasia, edema, or atrophy in the organs. The changes in organ weight did not differ from that in the vehicle group ($P > 0.05$) (Table 4).

Discussion

The agent sCT is used to treat metabolic bone disease, such as osteoporosis and Paget's disease, hypercalcemia, osteoarthritis, and bone-associated pain conditions.[9] The treatment entails high cost and therefore poor compliance in patients under long-term treatment. The development of a dosage form that improves the peptide and protein action via the gastrointestinal tract is one of the greatest challenges in the pharmaceutical field.[31] Cell engineering technology with recombinant microorganisms has created new opportunities in the development of innovative drugs. Although many microorganisms are potential hosts for the production of "biodrugs," yeast is a convenient candidate mainly because of its eukaryotic status and high level of resistance to gastric and pancreatic secretions.[18]

In this study, we successfully constructed a recombinant *S. cerevisiae* for low manufacturing cost and safe oral delivery of sCT by a yeast-surface-display system.

First, to prepare a biodrug with low manufacturing cost, yAGA2-sCT, we genetically engineered yeasts that were capable of serving as hosts for oral delivery of sCT in the digestive tract. The transformant yAGA2-sCT anchoring sCT can be harvested in large amounts at the stationary growth phase and freeze-dried for oral delivery of sCT without the purification and packing processes. The advantages of this approach may enlarge the application field of sCT. However, successful display on the cell surface is vital for this sCT biodrug. To stably display more sCT protein on the cell surface, the codon of sCT was optimized to adapt to expression in *S. cerevisiae*, and the original plasmid of pYD1 was modified into an integrating vector by inserting the *S. cerevisiae URA3* gene into both ends of the expression cassette. Therefore, nearly two-thirds of cells displayed sCT and one-half of cells V5, the control. However, Zhu et al.[32] used a yeast-surface-display system (EBY100 and pYD1) to express hemolysin from *Vibrio harveyi* and found only one-third (the largest amount) of the cells displaying hemolysin. This finding might be attributed to a higher stability of the integration of sCT or V5 expression cassettes in the host chromosomes than the free plasmid of pYD1.

Table 2. Hematological Parameters After 30-day Oral Gavage Administration of yAGA2-sCT to Rats

Group	Hemoglobin	Red Blood Cell Count	Platelet Count	White Blood Cell Count	Lymphocytes	Neutrophils
Vehicle group	101.9 ± 8.5	5.1 ± 0.5	444.3 ± 103.9	2.5 ± 0.9	89.1 ± 3.4	1.0 ± 0.3
0.1 g yAGA2-sCT	100.0 ± 11.0	5.1 ± 0.7	412.6 ± 115.2	3.1 ± 0.6	89.5 ± 4.1	1.2 ± 0.7
0.5 g yAGA2-sCT	100.2 ± 11.1	5.1 ± 0.6	453.8 ± 99.3	2.9 ± 1.0	88.7 ± 3.2	1.6 ± 0.8
5 g yAGA2-sCT	100.9 ± 8.0	5.2 ± 0.5	450.9 ± 107.5	2.6 ± 0.9	89.7 ± 2.5	1.1 ± 0.4

Table 3. Serum Levels of Chemical Parameters After 30-day Oral Gavage Administration of yAGA2-sCT to Rats

Parameter Level	Vehicle Group	0.1 g yAGA2-sCT	0.5 g yAGA2-sCT	5 g yAGA2-sCT
Alanine aminotransferase (U/L)	41.1 ± 6. 7	40.6 ± 5.9	42.0 ± 4.7	39.4 ± 5.0
Aspartate aminotransferase (U/L)	221.5 ± 58.2	228.1 ± 59.1	248.3 ± 61.7	218.3 ± 59.4
Blood urea nitrogen (mmol/L)	6.9 ± 0.5	6.5 ± 0.8	6.2 ± 1.0	7.0 ± 0.8
Cholesterol (mmol/L)	0.9 ± 0.5	1.2 ± 0.5	1.2 ± 0.2	1.3 ± 0.2
Triglycerides (mmol/L)	0.6 ± 0.3	0.8 ± 0.1	0.9 ± 0.2	1.1 ± 0.4
Creatinine (μmol/L)	27.8 ± 3.1	28.5 ± 2.1	28.6 ± 4.2	30.4 ± 2.3
Glucose (mmol/L)	2.3 ± 0.4	2.5 ± 0.7	2.3 ± 0.5	2.6 ± 0.7
Albumin (g/L)	31.7 ± 3.3	30.3 ± 1.3	32.2 ± 4.3	31.8 ± 1.4
Total protein (g/L)	75.2 ± 3.9	75.2 ± 3.9	80.8 ± 8.0	78.3 ± 2.5

Table 4. Ratio of Organ Weight to Final Body Weight of Rats Administered yAGA2-sCT

Parameter	Vehicle Group	0.1 g yAGA2-sCT	0.5 g yAGA2-sCT	5 g yAGA2-sCT
Heart	0.40 ± 0.04	0.40 ± 0.02	0.40 ± 0.06	0.38 ± 0.05
Lung	0.67 ± 0.07	0.64 ± 0.09	0.61 ± 0.08	0.65 ± 0.07
Liver	3.04 ± 0.17	3.15 ± 0.11	2.86 ± 0.15	3.04 ± 0.17
Spleen	0.30 ± 0.03	0.31 ± 0.02	0.29 ± 0.03	0.28 ± 0.03
Kidney	0.67 ± 0.03	0.70 ± 0.03	0.68 ± 0.06	0.67 ± 0.06
Testicle	1.07 ± 0.06	1.12 ± 0.07	1.14 ± 0.04	1.09 ± 0.11

Second, in vitro and in vivo, yAGA2-sCT showed bioactivity similar to that of the commercial product of sCT, Miacalcic. In vitro, the recombinant sCT inhibited osteoclastic bone resorption and decreased the area of lacuna for osteoclasts in the bone-wafer substrate (Figure 4). In vivo, oral administration of 5 g/kg yAGA2-sCT strain induced an hypocalcemic effect in rats. Interestingly, such oral administration induced a long-term hypocalcemic effect (Figure 5), as shown by the concentration–time profiles of ionized calcium in serum, with oral administration of 5 g/kg lyophilized yAGA2-V5 producing less change in serum calcium concentration over time than that with other treatments in rats. When nanoparticles are used as a carrier for sCT, a constant hypocalcemic effect will often be observed.[13] The prolonged hypocalcemic effect of yAGA2-sCT strain might be ascribed to a slow release of sCT from *S. cerevisiae*. Two reasons can explain the long-acting phenomenon of the recombinant sCT. One reason might be that an enterokinase site in the sCT expression cassette, no doubt, is convenient for the controlled release of sCT anchoring on the cell surface in that the enterokinase widely exists in the intestinal tract of many animals including humans.[33] The other reason might be the abundant glucan distributing on the yeast surface, which had high resistance to the digestive juice in the rats to protect sCT from degradation. The slow degradation of the recombinant sCT on the *S. cerevisiae* surface may achieve a longer period of sCT release, and thus resulting in a longer-acting hypocalcemia. This finding was similar to those with controlled release of other DDSs, such as basic fibroblast growth factor and bone morphogenetic protein 2 incorporated in self-assembled peptide-amphiphile nanofibers.[5,34] However, the effective dosage of 5 g/kg yAGA2-sCT seemed to be a little high for oral delivery. To resolve this situation, the efficiency of unit content of yAGA2-sCT can be improved by increasing the expression of sCT on yAGA2-sCT by selecting the recombinant of multiple site integration or tandem expression of sCT genes or by improving the amidation degree of the recombinant sCT, which is important to sCT activity, by coupling the production of sCT and amidating enzyme.[35] In this study, one glycine was added at the C terminus as an amidation site when the sCT gene was designed.

Third, the safety aspect of yAGA2-sCT was confirmed by the animal experiment. Before being integrated into the *S. cerevisiae* genome, the antibiotic marker had been deleted from the sCT expression cassette that originated from plasmid M-pYD1 for the sake of product safety. Therefore, the sCT biodrug made from the recombinant yAGA2-sCT did not contain any antibiotic products. However, *S. cerevisiae* is generally recognized as being safe and has recently been used as a probiotic in both animals and humans and as biotherapeutic agents in some human digestive pathologies, such as antibiotic-associated diarrhea and *Clostridium difficile* disease.[36] We did not observe any adverse effect of yAGA2-sCT on body weight, serum levels of chemical parameters, hematological parameters, or organ weight of the rats (Figure 6 and Tables 1–3).

In conclusion, the constructed yAGA2-sCT is a potential and safe biodrug but still needs further improvements for future application.

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