Substitution for Apoprotein of Neocarzinostatin by Self-Aggregate of Cholesterol-Bearing Pullulan

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A nanoparticle of cholesterol-bearing pullulan (CHP) self-aggregates effectively complexed with a neocarzinostatin chromophore (NCS-chr) in 0.5 M (1 M=1 mol dm^{-3}) AcONa/AcOH (pH 4.7) at 2 °C in the dark. The complex so obtained was very colloidally stable even after 4 d at 37 °C in a weak acidic medium. The complexed NCS-chr still maintained in vitro cytotoxicity to HeLa, HepG2, and 3′-mRLh-2 cells. In all cases, the cytotoxicity of the complex was higher than that of free NCS-chr itself. When the galactose moiety was additionally conjugated to CHP, the modified complex showed high cytotoxicity to a 3′-mRLh-2 cell. This cytotoxicity of the complex was almost comparable to that of intact NCS, which is ascribed to the galactose specific receptor-mediated endocytosis of the modified complex. This is the first finding to show that the complexed NCS-chr so obtained still maintains the strong cytotoxicity in vitro, and that the self-aggregate of hydrophobized polysaccharide (HPS) can nicely substitute for the apoprotein of NCS.

Neocarzinostatin (NCS) consists of a single polypeptide chain of 113-amino acids (apo-NCS) and non-protein chromophore (NCS-chr),[1] and is a potent macromolecular antitumor antibiotic.[2] The mechanism of biological activity of NCS has been extensively studied by Goldberg and coworkers.[3] Today it is well understood that NCS-chr is responsible for the biological activity, while apo-NCS has no cytotoxic activity at all.[4] NCS-chr itself is chemically and biochemically very unstable because of its labile bicyclic enediyne moiety, and easily loses its biological activity due to some external stimuli, such as light, heat, and existence of thiols, molecular oxygen, and bases (pH≥6).[5] Therefore, the most important function of apo-NCS is believed to be as a stabilizer of NCS-chr. Though many efforts have been made to overcome this disadvantage of NCS, only a few successful results have been encountered; for example, the chemical conjugation of NCS to copoly(styrene-*alt*-maleic anhydride) (SMANCS)[6] enhances the cytotoxic activity against tumors and reduces the side effect by the improved EPR effect.[7]

Since 1982, on the other hand, we have extensively studied the solution behavior of hydrophobized polysaccharides (HPS), such as choresteryl group-conjugated pullulan (CHP), and observed the formation of hydrogel nanoparticles upon the self-aggregation of the HPS in water (Fig. 1).[8] Interestingly, the hydrogel nanoparticles so obtained undergo spontaneous complexation with various hydrophobic substances[9] and/or soluble proteins and enzymes.[10,11] Very recently, we found that CHP also spontaneously complexes with NCS-chr and can nicely substitute for apo-NCS.[12] We also revealed that the NCS-chr/CHP complex so obtained still maintains activity to cleave the DNA strand of pBR322.[12] This is a sort of a biosimulation of the function of apoprotein with a

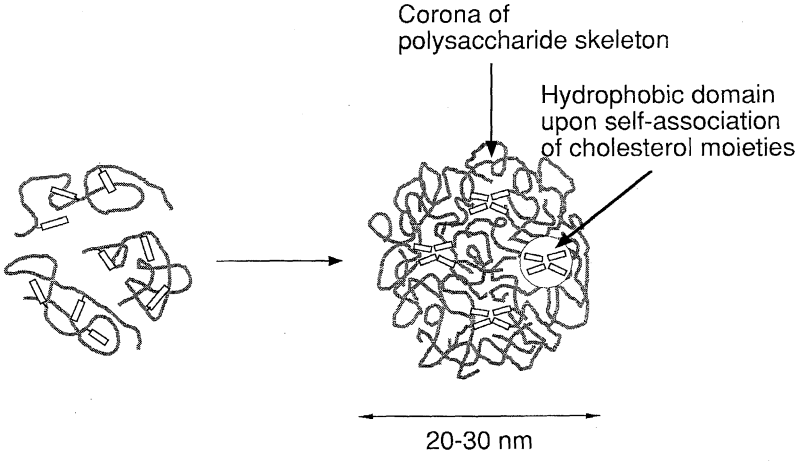


Fig. 1. Schematic drawing of a hydrogel nanoparticle formed by the self-aggregation of hydrophobized polysaccharide.

simple nonprotein macromolecule. On the other hand, we have also succeeded to make the HPS cell specific by additionally conjugating a saccharide determinant to CHP as the cell recognition site.[8)]

To further investigate whether the NCS-chr/HPS complex shows even in vitro cytotoxicity, and whether the hydrogel nanoparticle can surely replace the apoprotein of NCS, in this work we studied the cytotoxic activity of the complex to several mammalian cancer cells, such as HeLa, HepG2, and 3′-mRLh-2 cells.

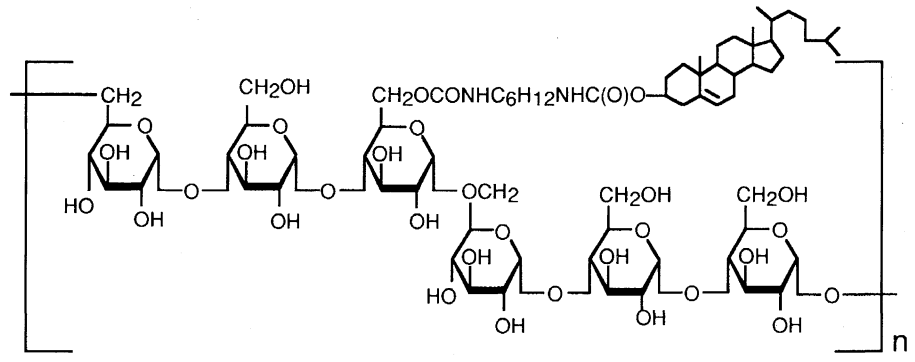
Results and Discussion

NCS and its polymeric prodrug (SMANCS) have been extensively employed for cancer chemotherapy.[2c)] NCS is a macromolecular antitumor antibiotic. Apo-NCS is a single-chain polypeptide that consists of 113 amino acid residues with an unusually high content of hydrophilic moieties.[13)] The native form of NCS is highly resistant to the action of proteinases, such as trypsin or chymotrypsin.[13)] On the other hand, NCS-chr is relatively hydrophobic because of the naphtonate moiety and the bicyclo[7.3.0]dodecadienediyne skeleton. In addition, NCS-chr has many sites capable of contributing to hydrogen-bonding formation.

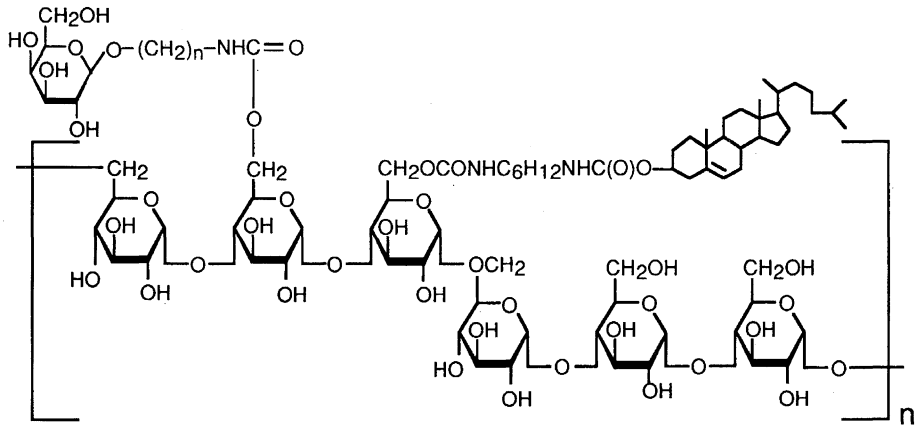
The HPSs undergo self-aggregation in water upon hydrophobic association of the conjugated hydrophobic anchor, such as the cholesteryl group, to give a hydrogel nanoparticle.[8)] With CHP-108-0.9, approximately 7 CHP molecules self-aggregate to give one nanoparticle, and one nanoparticle consists of approximately 10 cross-linking points as provided by hydrophobic association of the 4-5 cholesteryl groups (Fig. 2).[9)] The radius of gyration (R_G) of the CHP self-aggregate was measured by the HPSEC method:[14)] 16.0 nm for CHP-108-0.9 and 13.7 nm for Gal-(2)-7-CHP.

Our previous study revealed that the hydrogel nanoparticle of the CHP self-aggregates undergoes effective and spontaneous complexation with NCS-chr, and can nicely substitute for apo-NCS.[12)] Such a supramolecular assembly could be ascribed to the amphiphilic nature of the host and guest of both molecules.

The top in Fig. 3 shows an elution diagram of the NCS-chr/CHP complex detected by the RI index, while the bottom one shows UV detection at 340 nm for the same sample. In the absence of NCS-chr, of course, the parent CHP does not show any UV absorption at 340 nm. However, when NCS-chr was present and the elution was monitored by UV, a peak was detected at around $R_t = 22$ min. This peak clearly overlapped with that of parent CHP detected by RI, strongly suggesting the formation of the NCS-chr/CHP complex. The small peak around $R_t = 40$ min was uncomplexed NCS-chr.



CHP-108-0.9



Gal(2)-7-CHP-108-0.9

Fig. 2. Chemical structures of cholesterol-bearing pullulan (CHP-108-0.9) and its derivative Gal(2)-7-CHP-108-0.9.

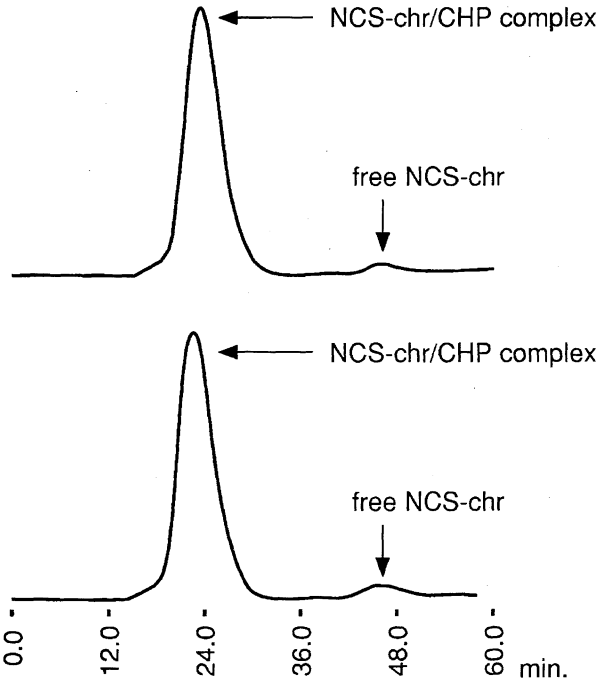


Fig. 3. Elution diagrams of a mixture of the CHP self-aggregate and NCS-chr monitored by RI detection (top) and UV detection at 340 nm (bottom). The elution was carried out at 22 °C with an aqueous 0.5 M AcONa/AcOH (pH 4.7) at a flow rate of 0.5 mL min^{-1}.

This result clearly suggests that we can easily separate free-NCS and the complex by the SEC technique. The radius of gyration of the HPS nanoparticles did not change much even after complexation: $R_G = 16.2$ nm for CHP-108-0.9 and 14.1 nm for Gal(2)-7-CHP. Under this condition, approximately 26 NCS-chr molecules could be complexed with one nanoparticle of CHP-108-0.9, while approximately 29 NCS-chr could be complexed with Gal(2)-7-CHP.

The NCS-chr/CHP complex so obtained was colloidally stable and did not aggregate or precipitate at all even after weeks. The particle size of the complex did not change at all during incubation at 37 °C (5% CO_2) for 4 d (Fig. 4) as well as after a freezing treatment. Moreover, no spontaneous leakage of NCS-chr from the complex was observed (Fig. 5).

As previously reported,[12] the complexed NCS-chr still kept its activity to cleave a double strand of DNA. In this work, to determine the maintenance of biological activity even in vitro, the cytotoxicity of the complex against several cancer cells was investigated (Fig. 6). With all of the cases, intact NCS certainly showed the highest cytotoxicity among them. On the other hand, free isolated NCS-chr almost lost its cytotoxicity under the conditions, and was comparable to the case of the control experiment. This means that NCS-chr is very unstable and easily loses its activity as previously reported.[4d,5] As expected, the NCS-chr/CHP complex showed a higher cytotoxicity than free NCS-chr, but much less than intact NCS with HeLa and HepG2 cells. This smaller cytotoxicity of the complex might be due to (1) a decreased cell uptake of the complex or (2) an actual deactivation of NCS-chr in the complex. To clarify this point,

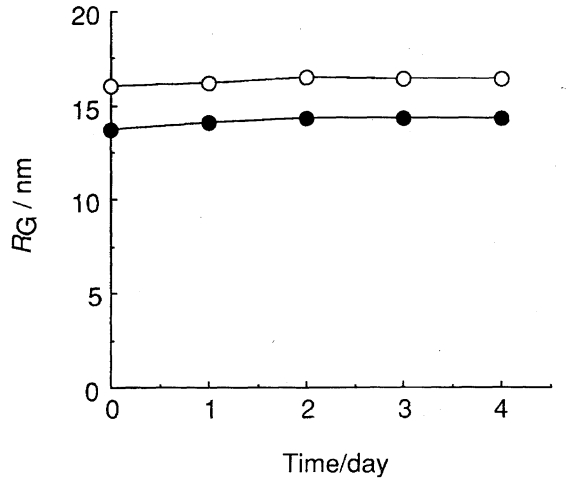


Fig. 4. Change in the radii of gyration (R_G) of the CHP (○) and Gal(2)-7-CHP (●) self-aggregates as a function of time in 0.5 M AcONa/AcOH (pH 4.7) at 37 °C under 5% CO_2.

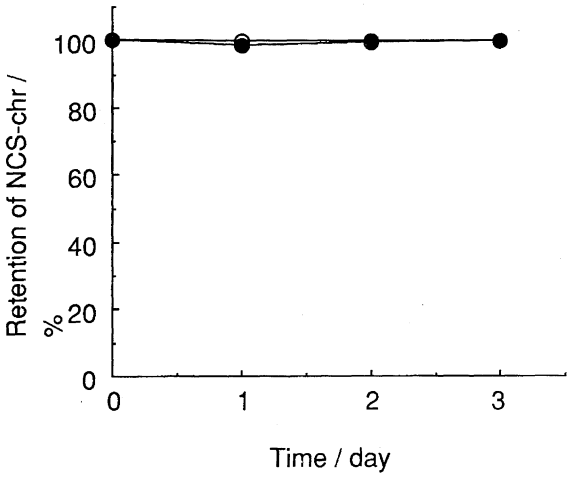


Fig. 5. Time course of leakage of NCS-chr from the complex as monitored by UV detection at 340 nm in 0.5 M AcONa/AcOH (pH 4.7) at 37 °C under 5% CO_2; for CHP (○), [saccharide] = 360 μg mL^{-1}, [NCS-chr] = 11.2 μM and Gal(2)-7-CHP (●), [HPS] = 360 μg mL^{-1}, [NCS-chr] = 11.6 μM.

we synthesized Gal(2)-7-CHP as described above. This galactose-moiety conjugated CHP is known to be specifically internalized by the cells which express a galactose-specific receptor on the cellular surface. All of the cancer cells employed in this work are known to bind more or less to galactoside-containing substances, and the binding affinity to the galactoside is the following: HeLa < HepG2≪3′-mRLh-2.[15,16] We then prepared the complex of NCS-chr with this modified HPS as usual, and studied its cytotoxicity to 3′-mRLh-2 cell (Fig. 6c). Surprisingly, when the galactose moiety was additionally conjugated to CHP, the cytotoxicity drastically increased and became almost comparable to that of intact NCS. Therefore, the results with HeLa and HepG2 cells can be ascribed not to the destruction of NCS-chr, but to the smaller cell uptake of the complex. The extent of the cytotoxicity against the three cancer cells was roughly par-

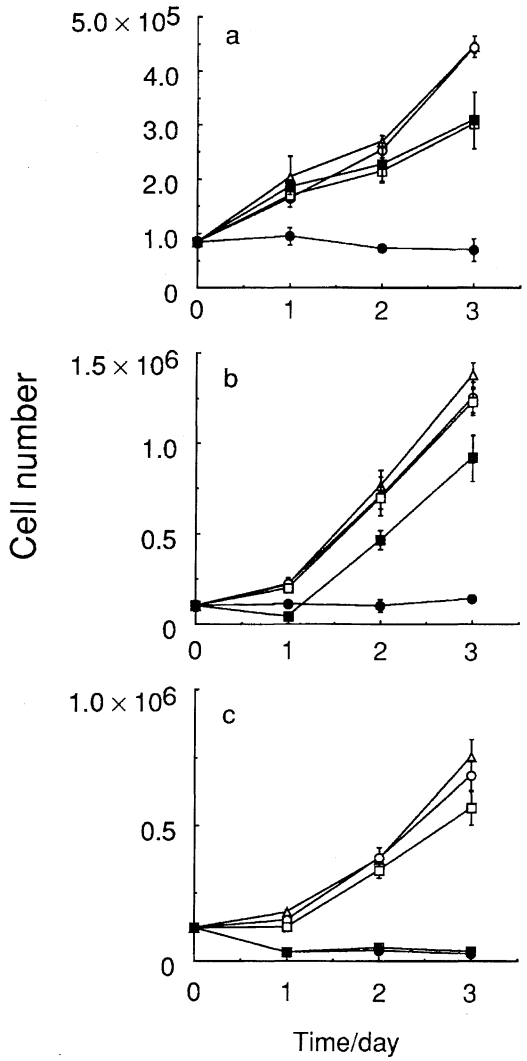


Fig. 6. Cytotoxicity of the NCS-chr/HPS to HeLa (a), HepG2 (b), and 3′-mRLh-2 cells (c) as a function of time at 37 °C under 5% CO_2; (△) control, (●) intact NCS, (○) NCS-chr, (□) NCS-chr/CHP, and (■) Gal-NCS-chr/CHP. Error bars denote ±SD ($n=4$). [NCS] = 90 nM (1.0 μg mL^{-1}), [NCS-chr] = 90 nM (60 ng mL^{-1}), [HPS] = 2.8 μg mL^{-1}, NCS-chr/one CHP nanoparticle = 26 (by mol) and NCS-chr/one Gal(2)-CHP nanoparticle = 29 (by mol).

allel to the sequence of the binding affinity or specificity of those cells to the galactoside. Another finding in this work is that intact NCS always showed a potent cytotoxicity, but less specificity to the three cancer cells studied. However, when we employed HPS as the stabilizer of NCS-chr or the substitute for apo-NCS, the cell specificity could be controlled. This is promising to diminish the side effect of NCS in antitumor treatments if we replace the apoprotein by an adequate HPS derivative that is specific to a target tumor cell.

In any event, the present results clearly suggest that the apoprotein of NCS is neither essential for NCS to show its cytotoxicity nor to be effectively internalized by tumor cells. Even if we substitute for the apoprotein by a suitable substance, we can still maintain the potent cytotoxicity of NCS-chr. In this sense, the present finding also suggests that the hydrogel nanoparticle is really promising to be a sort of site-specific drug carrier.

Experimental

CHP-108-0.9 (Fig. 2a) (Nippon Oil & Fat Co., Ltd., Tokyo, Japan) was exactly the same as that used in our previous work.[9] 2-Aminoethyl galactoside was synthesized as follows. Briefly, the hydroxy groups of D-galactose were protected by acetylation with acetic anhydride (yield, 32.2%). The tetraacetylgalactose so obtained was reacted with hydrobromic acid (yield, 47.9%) and then with 2-(*Z*-amino)ethanol (yield, 74.0%). 2-(*Z*-amino)ethyl tetraacetylgalactoside was then reduced by H_2 on Pd/C catalyst (yield, 70.0%). ^{1}H NMR ($CDCl_3$, 30 °C, 400 MHz) $\delta = 1.95$—2.20 ($COCH_3$), 3.29 ($OCH_2CH_2NH_2$), 3.34 (NH_2), 4.00 (*H*-5), 4.08—4.25 (*H*-6), 4.43 ($OCH_2CH_2NH_2$), 4.62 (*H*-1), 5.05 (*H*-3), 5.16 (*H*-2), and 5.41 (*H*-4). Detailed procedures of the subsequent conjugation of the galactosyl moiety to CHP-108-0.9 are described elsewhere.[17] Briefly, 2-aminoethyl galactoside obtained as mentioned above was conjugated to CHP-108-0.9 using the *p*-nitrophenyl chloroformate activation method.[18] The averaged substitution degree of the galactose moiety to parent CHP-108-0.9 was 7 per 100 anhydrous glucoside units of pullulan. This is coded as Gal(2)-7-CHP (Fig. 2b). Neocarzinostatin (NCS) was a kind gift of Kayaku Antibiotics Co., Ltd. (Tokyo, Japan). HeLa and 3′-mRLh-2 cells were purchased from Health Science Research Resources Bank (HSRRB) (Osaka, Japan), and HepG2 cells were a kind gift of Dr. M. Yamamoto (Department of the 2nd Surgery, School of Medicine, Nagasaki University). Minimum essential medium (MEM) and Dulbecco's minimum essential medium (DMEM) were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). DM-160 culture medium was obtained from Kyokuto Seiyaku Kogyo Co., Ltd. (Tokyo, Japan). Other chemicals were analytical grade and used without further purification.

Preparation of CHP Self-Aggregate Suspension. A 20 mg powder of CHP-108-0.9 or Gal(2)-7-CHP was added to 10 mL of 0.5 M AcONa/AcOH buffer (pH 4.7), and the polymer was well swelled for 3 d at 43 °C on a hot plate with a magnetic stirrer. The resulting suspension was sonicated using a probe-type sonifier (TOMY, UR-200P, Tokyo, Japan) for 5 min at 40 W and room temperature. Any impurities in the suspension were removed by filtering through a membrane filter (pore size 0.2 μm, Gelman Sciences Japan Ltd., Tokyo, Japan). The precise concentration of the polysaccharide was determined by the phenol–sulfuric acid method.[19]

The size of the hydrogel nanoparticle was determined on a high-performance size exclusion chromatography (HPSEC) (Shimadzu, Kyoto, Japan) using a Superose® 6HR10/30 column (Pharmacia Biotech, Uppsala, Sweden) connected to a RID-6A RI detector. The calibration was made using a standard sample kit of pullulan (Showa Denko, Tokyo, Japan).

Extraction of NCS-chr from NCS. The isolation of NCS-chr from NCS was carried out by exactly the same method as that used in previous work.[4a,12,20] Briefly, a 40 mg powder of NCS was suspended in 2.0 mL of 0.5 M AcOH/MeOH solution under shaking on a Vortex mixer, and the suspension was then centrifuged at 5,000×g and 4 °C for 5 min. The supernatant containing free NCS-chr was concentrated two fold by evaporation on a vacuum rotary evaporator. The NCS-chr concentration was spectrophotometrically determined: $\varepsilon_{340} = 1.08 \times 10^4$ M^{-1} cm^{-1}. All procedures were carried out in the dark.

Complexation between NCS-chr and Self-Aggregate of HPS. The complexation between the two was carried out by basically the same method as that employed before.[12] Briefly, 333 μL of 50 μM NCS-chr solution freshly prepared in 0.5 M AcOH/MeOH

was added dropwise to 3.0 mL of the polymer suspension in the same buffer (2.0 mg mL^{-1}). The resulting mixture was incubated for 24 h at 2 °C in the dark, and uncomplexed free NCS-chr was then isolated gel-chromatographically by HPSEC (Tosoh Co., Ltd., Tokyo, Japan) using a Superose® 6HR 10/30 prepacked column, a CCPS dual pump, a RI-8020 RI detector, and a UV-8020 UV-visible detector. The sample solution was eluted out at 22 °C with 0.5 M AcONa/AcOH buffer (pH 4.7) at a flow rate of 0.5 mL min^{-1}. The spontaneous dissociation of NCS-chr from the complex was monitored by the same HPSEC method.

Cytotoxicity of NCS-chr/HPS Complex. The cancer cells studied were maintained in an adequate medium for each cell: MEM for HeLa cell, DMEM for HepG2 cell, and DM-160 for 3′-mRLh-2 cell, respectively, at 37 °C in 5% CO_2 incubator. The MEM medium was supplemented with 10 vol% heat-inactivated fetal bovine serum (FBS) and 292 μg mL^{-1} of L-glutamate. The DMEM and DM-160 media were supplemented with 10 vol% heat-inactivated FBS, 100 units mL^{-1} of penicillin, and 0.12 mcg mL^{-1} of streptomycin.

Cells of the growth phase were treated with a 0.05% trypsin-0.025% EDTA aqueous solution and suspended in the basal medium. A 2.0 mL cell suspension was seeded on a 6-well microtiter plate (SUMILON, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) at a density of 4×10^4 cells mL^{-1}. After pre-incubation for 24 h at 37 °C in 5% CO_2, the cells were treated with 50 μL of the complex solution. A control experiment was made using the same buffer (pH 4.7) without any cytotoxic agent. On appropriate days, the supernatant was removed, and the cell pellets were washed twice with 2 mL of 150 mM phosphate buffered saline (PBS) (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 followed by a trypsin treatment. The living cells were counted by the erythrosine B staining method on a hemocytometer.

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