

NOVEL ARTIFICIAL PROTEIN CATALYST

Technical Field

[0001]

The present invention relates to a novel artificial protein catalyst.

Background Art

[0002]

Currently, attempts related to "*in vivo* synthetic chemical treatments" utilizing catalysts are being investigated. The concept of *in vivo* synthetic chemical treatment is to introduce a material or reagent without activity or toxicity into the body, and activating the said material or reagent at a particular location in the body with a catalyst to allow effects to be expressed. In such circumstances, interests related to the development of a new catalyst that is applicable to therapeutic applications are increasing (Non-Patent Literature 1).

[0003]

One obstacle in the development of such catalyst is the protection of a catalyst from *in vivo* substances. For example, it is known that metal catalysts (metalloenzymes) such as gold (Au), palladium (Pd), ruthenium (Ru), and the like are quickly inactivated when exposed to thiol-containing glutathione (GSH) that exists in cells in the range of 0.5 - 10 mM or in blood plasma in the range of about 2 - 20 μ M.

[0004]

At this point, in many research, investigation of catalyst reaction has only been performed in cells or in models other than mammals such as zebrafish via any of artificial metal enzymes or metal catalyst complexes (Non-Patent Literatures 2 - 17).

Citation List

Non-Patent Literatures

[0005]

- [Non-Patent Literature 1] Rebelein, J. G.; Ward, T. R. *Curr. Opin. Biotechnol.* 2018, 53, 106-114.
- [Non-Patent Literature 2] Miller, M. A.; Askevold, B.; Mikula, H.; Kohler, R. H.; Pirovich, D.; Weissleder, R. *Nat. Commun.* 2017, 8, 15906.
- [Non-Patent Literature 3] Clavadetscher, J.; Hoffmann, S.; Lilienkampf, A.; Mackay, L.; Yusop, R. M.; Rider, S. A.; Mullins, J. J.; Bradley, M. *Angew. Chem. Int. Ed. Engl.* 2016, 55, 15662-15666.
- [Non-Patent Literature 4] Clavadetscher, J.; Indrigo, E.; Chankeshwara, S. V.; Lilienkampf, A.; Bradley, M. *Angew. Chem. Int. Ed. Engl.* 2017, 56, 6864-6868.
- [Non-Patent Literature 5] Weiss, J. T.; Dawson, J. C.; Macleod, K. G.; Rybski, W.; Fraser, C.; Torres-Sanchez, C.; Patton, E. E.; Bradley, M.; Carragher, N. O.; Unciti-Broceta, A. *Nat. Commun.* 2014, 5, 3277.
- [Non-Patent Literature 6] Perez-Lopez, A. M.; Rubio-Ruiz, B.; Sebastian, V.; Hamilton, L.; Adam, C.; Bray, T. L.; Irusta, S.; Brennan, P. M.; Lloyd-Jones, G. C.; Sieger, D.; Santamaria, J.; Unciti-Broceta, A. *Angew. Chem. Int. Ed. Engl.* 2017, 56, 12548-12552.
- [Non-Patent Literature 7] Bray, T. L.; Salji, M.; Brombin, A.; Perez-Lopez, A. M.; Rubio-Ruiz, B.; Galbraith, L. C. A.; Patton, E. E.; Leung, H. Y.; Unciti-Broceta, A. *Chem. Sci.* 2018, 9, 7354-7361.
- [Non-Patent Literature 8] Liu, Y.; Pujals, S.; Stals, P. J. M.; Paulohrl, T.; Presolski, S. I.; Meijer, E. W.; Albertazzi, L.; Palmans, A. R. A. *J. Am. Chem. Soc.* 2018, 140, 3423-3433.
- [Non-Patent Literature 9] Li, J.; Yu, J.; Zhao, J.; Wang, J.; Zheng, S.; Lin, S.; Chen, L.; Yang, M.; Jia, S.; Zhang, X.; Chen, P. R. *Nat. Chem.* 2014, 6, 352-361.
- [Non-Patent Literature 10] Vidal, C.; Tomas-Gamasa, M.; Destito, P.; Lopez, F.; Mascarenas, J. L. *Nat. Commun.* 2018, 9, 1913.
- [Non-Patent Literature 11] Destito, P.; Sousa-Castillo, A.; Couceiro, J. R.; Lopez, F.; Correa-Duarte, M. A.; Mascarenas, J. L. *Chem. Sci.* 2019, 10, 2598-2603.

[Non-Patent Literature 12] Tonga, G. Y.; Jeong, Y.; Duncan, B.; Mizuhara, T.; Mout, R.; Das, R.; Kim, S. T.; Yeh, Y.-C.; Yan, B.; Hou, S.; Rotello, V. M. *Nat. Chem.* 2015, 7, 597-603.

[Non-Patent Literature 13] Streu, C.; Meggers, E. *Angew. Chem. Int. Ed. Engl.* 2006, 45, 5645-5648.

[Non-Patent Literature 14] Volker, T.; Dempwolff, F.; Graumann, P. L.; Meggers, E. *Angew. Chem. Int. Ed. Engl.* 2014, 53, 10536-10540.

[Non-Patent Literature 15] Tomas-Gamasa, M.; Martinez-Calvo, M.; Couceiro, J. R.; Mascarenas, J. L. *Nat. Commun.* 2016, 7, 12538.

[Non-Patent Literature 16] Yusop, R. M.; Unciti-Broceta, A.; Johansson, E. M. V.; Sanchez-Martin, R. M.; Bradley, M. *Nat. Chem.* 2011, 3, 239-243.

[Non-Patent Literature 17] Unciti-Broceta, A.; Johansson, E. M. V.; Yusop, R. M.; Sanchez-Martin, R. M.; Bradley, M. *Nat. Protoc.* 2012, 7, 1207-1218.

Summary of the Invention

Problems to be Solved by the Invention

[0006]

The object of the present invention is to provide a novel artificial protein catalyst that allows the protection of a catalyst from *in vivo* substances and has potential usefulness for *in vivo* synthetic chemical treatment.

Means for Solving the Problems

[0007]

Human serum albumin (HSA) is a 66.5 kDa protein that exists abundantly in blood plasma, and it is known to have a half-life in the serum of about 19 days (Peters, T., Jr. *Adv. Protein Chem.* 1985, 37, 161-245.). HSA is known to be a carrier protein for various hormones, fatty acids, and low-molecular agents, and multiple major and minor binding sites for numerous kinds of ligands have been confirmed in HSA (Ghuman, J.; Zunszain, P. A.; Petitpas, I.; Bhattacharya, A. A.; Otagiri, M.; Curry, S. J. *Mol. Biol.* 2005, 353, 38-52.).

[0008]

In order to design biocompatible artificial protein catalysts, the present inventors selected HSA as the protein, and considered accommodating a metal catalyst in the hydrophobic binding pocket of HSA. It was found that by using a metal catalyst ruthenium as the catalyst, when the metal catalyst was fixed to the albumin drug binding site I known for binding (interaction) with a coumarin derivative (such as warfarin), it was possible to protect the catalytic activity of the bound ruthenium under *in vitro* conditions even in the presence of 20 mM GSH, thus coming to complete the present invention.

[0009]

In other words, the present invention encompasses the following characteristics:

[1] A complex of a protein and a catalyst selected from metal catalysts or organic catalysts, wherein

said protein is a protein that has a hydrophobic pocket within its three-dimensional structure, and

said complex accommodates said catalyst in said hydrophobic pocket so that said catalyst is not exposed or not substantially exposed to the hydrophilic environment.

[0010]

[2] The complex according to [1], wherein said protein is a natural or artificial protein.

[0011]

[3] The complex according to [1] or [2], wherein said protein is selected from the group consisting of human serum albumin (HSA), immunoglobulin G (IgG), immunoglobulin A (IgA), transferrin, antitrypsin, haptoglobin, α 1-acidic glycoprotein, Myoferlin, Trk receptor, estrogen receptor, and folate receptor.

[0012]

[4] The complex according to any of [1] to [3], wherein said metal catalyst is selected from the group consisting of a boron catalyst, a magnesium catalyst, an aluminum catalyst, a silicon catalyst, a calcium catalyst, a scandium catalyst, a titanium catalyst, a vanadium catalyst, a chromium catalyst, a manganese catalyst, an iron catalyst, a cobalt catalyst, a nickel

catalyst, a copper catalyst, a zinc catalyst, an yttrium catalyst, a zirconium catalyst, a niobium catalyst, a molybdenum catalyst, a ruthenium catalyst, a rhodium catalyst, a palladium catalyst, a silver catalyst, an indium catalyst, a tin catalyst, a barium catalyst, a hafnium catalyst, a tungsten catalyst, a rhenium catalyst, an osmium catalyst, an iridium catalyst, a platinum catalyst, a gold catalyst, and a lanthanoid Lewis acid catalyst.

[0013]

[5] The complex according to [4], wherein said lanthanoid Lewis acid catalyst is selected from the group consisting of an ytterbium catalyst, a lanthanum catalyst, a cerium catalyst, a samarium catalyst, an europium catalyst, a gadolinium catalyst, a terbium catalyst, a thulium catalyst, and a lutetium catalyst.

[0014]

[6] The complex according to any of [1] to [5], wherein said protein is a human serum albumin, and said catalyst is a metal catalyst.

[0015]

[7] The complex according to [6], wherein said metal catalyst is a ruthenium catalyst.

[0016]

[8] The complex according to [6] or [7], wherein said hydrophobic pocket of human serum albumin is the albumin drug binding site I (drug site I).

[0017]

[9] The complex according to any of [6] to [8], wherein said metal catalyst is bound to the said hydrophobic pocket via a ligand against human serum albumin.

[0018]

[10] The complex according to [9], wherein said ligand is selected from the group consisting of warfarin, azapropazone, acenocoumarol, phenylbutazone, salicylate salt, indomethacin, phenytoin, tolbutamide, chlorpropamide, iophenoxate, iodipamide, sulfadimethoxine, phenprocoumon, glibenclamide, sulfathiazole, tenoxicam, camptothecin, balzidendeazi, andelleratan, diadelleal,

diadellealtan, prodan, bilirubin, eicosanoid, and carboxy-methyl-propyl-furanpropanoate (uremic toxin), as well as coumarin.

[0019]

[11] The complex according to [9] or [10], wherein said metal catalyst is bound to the said hydrophobic pocket via a linker bound to said ligand.

[0020]

[12] The complex according to [11], wherein said linker is an alkyl chain or a polyethylene glycol (PEG) chain having amino and carboxyl groups on both ends.

[0021]

[13] The complex according to [12], wherein said linker is a C₁ - C₃ alkyl or a PEG chain with a polymerization degree of 1 - 3.

[0022]

[14] The complex according to any of [1] to [13], wherein the surface of said protein is modified so as to interact with a target site *in vivo*.

[0023]

[15] The complex according to [14], wherein said modification is a modification by a sugar chain.

[0024]

[16] The complex according to any of [1] to [13], wherein said protein further comprises a portion that interacts with a target site *in vivo*.

[0025]

[17] The complex according to [14] or [15], wherein said protein is human serum albumin.

[0026]

[18] A composition comprising the complex according to any of [1] to [17].

[0027]

[19] The composition according to [18], which is a pharmaceutical composition further comprising a pharmaceutically acceptable carrier.

[0028]

[20] The composition according to [19], which is used in combination with a prodrug that can be activated by said complex.

[0029]

[21] The composition according to [19], further comprising a prodrug that can be activated by said complex.

[0030]

[22] The composition according to [19], which is employed for selectively tagging particular cells.

[0031]

[23] The composition according to [22], which is administered in combination with a chemical substance that is tagged to said cells.

[0032]

[24] The composition according to [18], which is used as a biosensor.

[0033]

[25] The composition according to [18], which is used as a biosensor for detecting ethylene.

[0034]

[26] A pharmaceutical composition comprising a prodrug, wherein

said prodrug can be activated by the complex according to any of [1] to [17], and

said pharmaceutical composition is used in combination with the complex according to any of [1] to [17].

[0035]

[27] A combination medicine comprising
a first agent comprising the complex according to any of [1] to [17], and

a second agent comprising a prodrug that can be activated by said complex.

[0036]

Those skilled in the art will be able to recognize that an invention of any combination of one or more characteristics of the present invention described above is also encompassed by the scope of the present invention.

Effects of the Invention

[0037]

According to the present invention, a novel artificial protein catalyst that allows the protection of a catalyst from *in vivo* substances is provided.

Brief Description of the Drawings

[0038]

[Figure 1] Figure 1 shows the fluorescence assay result of the product obtained by the reaction between ruthenium catalysts Ru1 - 3, 6 and HSA.

[Figure 2] Figure 2 show the saturated binding curves of performing reaction between HSA and warfarin or ibuprofen, and then reaction with ruthenium catalysts Ru1 - 3, 6.

[Figure 3] Figure 3 shows the experimental system for testing the catalytic ability of alb-Ru complex employed in the Examples herein.

[Figure 4] Figure 4 shows the evaluation result of the catalytic ability of alb-Ru complex under a different condition.

[Figure 5] Figure 5 shows the evaluation result of the catalytic ability of alb-Ru complex under a different condition.

[Figure 6] Figure 6 shows the Michaelis-Menten kinetic parameters of substrates 1a - e in the presence or absence of GSH in regards to ArM activity (artificial metal enzyme activity).

[Figure 7] Figure 7 shows the molecular modeling of when ruthenium catalysts Ru1 - 3, 6 are docked with human serum albumin (PDB:1H9Z).

[Figure 8] Figure 8 is cell imaging results showing that GArM-Ru1(2,3-Sia) is better accumulated in SW620 human colon adenocarcinoma cells.

[Figure 9] Figure 9 shows the comparison between SW620 human colon adenocarcinoma cells, HeLa human cervical cancer-derived cells, and A549 human pulmonary alveolar basement epithelial adenocarcinoma cells in regards to accumulation of GArM-Ru1(2,3-Sia).

[Figure 10] Figure 10 shows a kinetic experiment in regards to the activation of prodrugs 1g and 1h by GArM-Ru complex.

[Figure 11] Figure 11 shows the comparison of cell growth inhibition effect on cancer cell strains by prodrug, prodrug and alb-Ru1, or prodrug and GArM-Ru1.

[Figure 12] Figure 12 shows the synthetic scheme of the ArM ethylene probe (AEP).

[Figure 13] Figure 13 shows the observed fluorescence intensity of alb-Ru compound compared to the AEP probe.

[Figure 14] Figure 14 shows the comparison of CD spectra for examining the change in the folding structure of various protein compounds in this research.

[Figure 15] Figure 15 shows the result of observing the change in the amount of ethylene produced expressed in various organelles (the outer pericarp, loculus, and columella) in different developmental stages (immature and mature) of kiwi fruits with AEP. The number of samples is the outer pericarp (n = 24), loculus (n = 2), and columella (n = 4), and statistical analysis was performed with a t-test of a pair of samples. *P < 0.03, **P < 0.002, ***P < 0.0002, ****P < 0.0001, ns = not essential.

[Figure 16] Figure 16 shows the fluorescence intensity and bright field microscope imaging images (40 X magnification) of the epidermis of wild-type Col-0 (b) that was applied AEP (100 μ M), as well as the results of further addition of an ethylene biosynthesis accelerator ACC (1 mM) and detection.

[Figure 17] Figure 17 shows the result of observing the amount of ethylene produced from *Arabidopsis thaliana* by addition of ACC with fluorescence intensity. All values were obtained in triplicate experiments. Statistical analysis was performed with one-way analysis of variance of Tukey's multiple comparison test. *P < 0.03, **P < 0.002, ***P < 0.0002, ****P < 0.0001, ns = not essential.

[Figure 18] Figure 18 shows the comparison of flow cytometry results of GArM-treated HeLa cells and untreated HeLa cells.

[Figure 19] Figure 19 shows the comparison of flow cytometry results of GArM-treated mouse peritoneal cavity-derived

macrophages and untreated mouse peritoneal cavity-derived macrophages.

[Figure 20] Figure 20 shows the comparison of flow cytometry results of HeLa cells and mouse peritoneal cavity-derived macrophages treated with GAR_M, as well as HeLa cells and mouse peritoneal cavity-derived macrophages not treated with GAR_M. Note that the Q3 values of the upper left and the bottom left figures are 99.09% and 43.06%, respectively.

[Figure 21] Figure 21 show the result of cell adhesion assay performed in Example 8.

Description of Embodiments

[0039]

The present invention relates to a novel artificial protein catalyst. Specifically, the artificial protein catalyst according to the present invention is a complex of a protein and a catalyst selected from metal catalysts or organic catalysts (hereinafter also referred to as "the complex of the present invention.")

[0040]

The complex of the present invention can be characterized by a configuration that accommodates a catalyst within the hydrophobic pocket of a protein that has said hydrophobic pocket within its three-dimensional structure, so that the catalyst is not exposed or not substantially exposed to the hydrophilic environment. In other words, the complex of the present invention can protect the catalyst from *in vivo* substances by avoiding exposure or substantial exposure of the catalyst to the hydrophilic environment. The complex of the present invention may accommodate the catalyst in only one out of the hydrophobic pockets present in the protein, or may accommodate the catalyst in multiple (or all) pockets.

[0041]

The complex of the present invention can also be characterized by having a configuration that avoids exposure or substantial exposure of the catalyst to the hydrophilic environment, and meanwhile the catalyst may still promote the

target reaction while having the aforementioned configuration. In other words, in a preferred embodiment, the complex of the present invention may protect the catalyst from *in vivo* substances while exerting the desired activity *in vivo*.

[0042]

In the present invention, "the catalyst is not substantially exposed to the hydrophilic environment" refers to that exposure to the hydrophilic environment is permitted to the extent that protection of the catalyst from the hydrophilic environment is recognized. "Protection of the catalyst from the hydrophilic environment is recognized" refers to that in comparison to when a free catalyst is exposed to an *in vivo* environment, the activity of the catalyst accommodated in the complex of the present invention is maintained for a longer period under the same environment, and for example can be evaluated by metabolic turnover (TON). The extent that the exposure is permitted may change depending on the type of catalyst used, or the type of protein used in combination, etc.

[0043]

In one embodiment of the present invention, "the catalyst is not substantially exposed to the hydrophilic environment" means that the relative solvent accessible surface area (SASA) of the catalyst when accommodated in the hydrophobic pocket of the protein is 5.0 or less, preferably 4.0 or less, more preferably 3.5 or less, more preferably 3.0 or less, more preferably 2.5 or less, more preferably 2.0 or less, more preferably, 1.5 or less, more preferably 1.0 or less, more preferably 0.9 or less, more preferably 0.8 or less, more preferably 0.7 or less, more preferably 0.6 or less, more preferably 0.5 or less, more preferably 0.4 or less, more preferably 0.3 or less, more preferably 0.2 or less, or more preferably 0.1 or less.

[0044]

In another embodiment of the present invention, "the catalyst is not substantially exposed to the hydrophilic environment" means that 50% or more, preferably 55% or more, more preferably 60% or more, more preferably 65% or more, more

preferably 70% or more, more preferably 75% or more, more preferably 80% or more, more preferably 85% or more, more preferably 90% or more, more preferably 95% or more, more preferably 96% or more, more preferably 97% or more, more preferably 98% or more, or more preferably 99% or more of the total surface area of the catalyst is accommodated internally in the hydrophobic pocket of the protein. Alternatively, "the catalyst is not substantially exposed to the hydrophilic environment" means that the area of the catalyst exposed to the hydrophilic environment is 50% or less, preferably 45% or less, more preferably 40% or less, more preferably 35% or less, more preferably 30% or less, more preferably 25% or less, more preferably 20% or less, more preferably 15% or less, more preferably 10% or less, more preferably 5% or less, more preferably 4% or less, more preferably 3% or less, more preferably 2% or less, or more preferably 1% or less of the total surface area of the catalyst.

[0045]

The protein that can be used for the complex of the present invention is not limited as long as it has one or more hydrophobic pockets that allow accommodation of the catalyst, and natural or artificial proteins can be employed. Artificial proteins include a mutated protein where a mutation is artificially introduced into a part of a natural protein.

[0046]

In one embodiment, the protein used for the complex of the present invention is a protein where a ligand that binds to or interacts with any of the hydrophobic pockets of the protein is easily obtained. Such a protein may be preferable in that it facilitates the complexation of the catalyst via the ligand.

[0047]

In a specific embodiment, the protein used for the complex of the present invention is a protein selected from human serum albumin (HSA), immunoglobulin G (IgG), immunoglobulin A (IgA), transferrin, antitrypsin, haptoglobin, α 1-acidic glycoprotein, and the like. These proteins are thought to be suitable for drug

delivery system since they are proteins that can move freely in blood. In yet another specific embodiment, the protein used for the complex of the present invention is a protein selected from Myoferlin, Trk receptor, estrogen receptor, folate receptor, and the like. Since these proteins are known to be greatly expressed in cancer cells, it is thought that by coordinating ligands and metals thereto, a complex that can directly kill cancer could be manufactured.

[0048]

The organic or metal catalyst that can be used for the complex of the present invention is not particularly limited, and can be arbitrary selected by those skilled in the art. In one embodiment, the organic or metal catalyst that can be used for the complex of the present invention is one that has the activity to change a given prodrug to an active form.

[0049]

For example, the organic catalyst used for the complex of the present invention can include, but is not limited to, proline derivatives, phase transfer catalysts based on quaternary ammonium salt derivatives, thiourea derivatives, N-heterocyclic carbene derivatives obtained from thiazolium salts or imidazolium salts, cyclic ketone derivatives, 4-dimethylaminopyridine derivatives, secondary amines such as amino acids, and the like.

[0050]

For example, the metal catalyst used for the complex of the present invention can include, but is not limited to, a boron catalyst, a magnesium catalyst, an aluminum catalyst, a silicon catalyst, a calcium catalyst, a scandium catalyst, a titanium catalyst, a vanadium catalyst, a chromium catalyst, a manganese catalyst, an iron catalyst, a cobalt catalyst, a nickel catalyst, a copper catalyst, a zinc catalyst, an yttrium catalyst, a zirconium catalyst, a niobium catalyst, a molybdenum catalyst, a ruthenium catalyst, a rhodium catalyst, a palladium catalyst, a silver catalyst, an indium catalyst, a tin catalyst, a barium catalyst, a hafnium catalyst, a tungsten catalyst, a rhenium catalyst, an osmium catalyst, an iridium catalyst, a platinum

catalyst, a gold catalyst, a lanthanoid Lewis acid catalyst, and the like. Moreover, said lanthanoid Lewis acid catalyst can also include, but is not limited to, an ytterbium catalyst, a lanthanum catalyst, a cerium catalyst, a samarium catalyst, an europium catalyst, a gadolinium catalyst, a terbium catalyst, a thulium catalyst, a lutetium catalyst, and the like.

[0051]

Means for accommodating the catalyst in the hydrophobic pocket of the protein can include, for example, a means for binding the catalyst into the hydrophobic pocket via a ligand that interacts with or binds to said hydrophobic pocket. In such a case, a linker of peptides, hydrocarbon chains, PEGs, and the like can be appropriately employed for linking the ligand and the catalyst (such as a metal catalyst). The length of the linker used should be a length such that the catalyst is accommodated within the hydrophobic pocket and is not exposed or not substantially exposed to the hydrophilic environment. In other words, when the linker used is too long in relation to the hydrophobic pocket, the ligand that interacts therewith, and the size of the catalyst used, there is a possibility that when the catalyst is bound to the hydrophobic pocket via the linker and the ligand, the catalyst is not housed within the hydrophobic pocket, all or a portion thereof is exposed from the hydrophobic pocket to the hydrophilic environment, and sufficient protection from the hydrophilic environment could not be obtained.

[0052]

Another means for accommodating the catalyst in the hydrophobic pocket of the protein can include, for example, applying maleimide or succinimide to the side chain functional group of cysteine or lysine (thiol group and amino group, respectively) present within or in the vicinity of the hydrophobic pocket of the protein to allow activation and covalent binding with the catalyst.

[0053]

Yet another means for accommodating the catalyst in the hydrophobic pocket of the protein can include introducing a

mutation in a particular position of the amino acid sequence that configures the hydrophobic pocket to introduce a double bond or an azide to allow metathesis or click reaction and covalent binding with the catalyst (Young TS, Ahmad I, Brock A, Schultz PG., Expanding the genetic repertoire of the methylotrophic yeast *Pichia pastoris*., Biochemistry. 2009. 48 (12): 2643-53.; Wang L, Schultz PG., Expanding the genetic code., Angew Chem Int Ed Engl. 2004. 44 (1): 34-66.). In such a case, a catalyst having a linker such as a peptide or PEG can be used as the catalyst (such as a metal catalyst).

[0054]

In one embodiment, the complex of the present invention is modified to interact with the *in vivo* target site. As a result, the complex of the present invention can be guided to an *in vivo* desired site or can target the desired site. In relation to this, "interacts with a target site *in vivo*" may mean accumulates or typically binds to the target site with significantly strong directionality compared to other *in vivo* sites. In the present invention, the target site may be a particular organ or a particular cytoma etc.

[0055]

Such modifications can include e.g. glycosylation. For example, it is known that by employing an asparagine-linked sugar chain (N-linked sugar chain) for protein modification, the directionality towards a particular organ changes depending on the number of clusters and/or the number of bindings of its sugar chain (Tanaka, K., et al., Angew. Chem. Int. Ed., 49, 8195-8200 (2010); Latypova, L., et al., Adv. Sci., 1600394 (2017); Ogura, A., et al., Chem. Commun., 54, 8693-8696 (2018); Taichi, M., et al., Adv. Sci., 1700147 (2017)). For example, it is known that a sugar chain having a sialic acid on its terminal swiftly reaches the liver, or a sugar chain having a galactose on its terminal the intestinal tract. Accordingly, by modifying the complex of the present invention with the appropriate sugar chain structure and number of sugar chains depending on the selected *in vivo* target

site, a complex with enhanced directionality towards said target site can be obtained.

[0056]

In one embodiment, for example, multiple (such as 5 - 30) sugar chains having sialic acid, galactosamine, galactose, or mannose that interact with various types of cancer at the non-reducing terminal can be employed as the glycosylation. In an alternative embodiment, sugar chains having fucose can be employed as the glycosylation. In the aforementioned embodiment, the multiple sugar chains to be modified may be the same or multiple types, and the size of each sugar chain can be in the range of from 1 to 25 sugars.

[0057]

In another embodiment, for example, multiple (such as 5 - 30) sugar chains having sialic acid, galactosamine, galactose, or mannose that selectively transfers into particular organs such as liver, pancreas, intestinal tract, gallbladder, bladder, or brain at the non-reducing terminal can be employed as the glycosylation. In the aforementioned embodiment, the multiple sugar chains to be modified may be of the same or multiple types, and the size of each sugar chain may be in the range of from 1 to 25 sugars.

[0058]

The modification site is typically the protein surface of said complex. The method for glycosylating a particular site of the protein surface is well-known to those skilled in the art, and any method may be employed. In the present invention, for example, glycosylation can be introduced to the protein surface by the click reaction described in International Publication No. 2008/096760, Japanese Patent No. 6327547, International Publication No. 2017/002918, and the like.

[0059]

In another embodiment, the complex of the present invention further comprises a portion that interacts with a target site *in vivo*. In relation to this, "interacts with the target site" may mean accumulates or typically binds to the target site with significantly strong directionality compared to other sites. In

the present invention, the target site may be a particular organ or a particular cytoma etc.

[0060]

In the present invention, "a portion that interacts with a target site *in vivo*" may be an antibody or a fragment thereof, a peptide ligand or a fragment thereof, DNA or RNA, or pNA (peptide nucleic acid) or a fragment thereof, and the like. In relation to this, "a portion that interacts with a target site *in vivo*" may for example be manufactured as a fusion protein with the protein employed for the complex, or the protein employed for the complex and "a portion that interacts with a target site *in vivo*" may be separately prepared and then bound (e.g. covalently bound) by means well-known to those skilled in the art.

[0061]

In a specific embodiment, the present invention relates to an artificial metal enzyme (artificial metalloenzyme; ArM) which is a complex of a protein and a metal catalyst. In a preferred embodiment, the ArM of the present invention is a complex of HSA and a metal catalyst, wherein said complex is characterized in that it accommodates the metal catalyst in the hydrophobic pocket of HSA so that the metal catalyst is not exposed or not substantially exposed to the hydrophilic environment.

[0062]

In this embodiment, the metal catalyst used together with HSA can be appropriately selected by those skilled in the art for example according to the type of prodrug subject to activation etc. In one embodiment of the present invention, the metal catalyst used together with HSA is ruthenium.

[0063]

In this embodiment, the hydrophobic pocket of HSA that accommodates the metal catalyst is not particularly limited, and one hydrophobic pocket may accommodate the metal catalyst or multiple hydrophobic pockets may accommodate the metal catalyst. The hydrophobic pocket of HSA can include the albumin drug binding site I and the albumin drug binding site II. In one embodiment,

the complex of the present invention accommodates the metal catalyst in the albumin drug binding site I of HSA.

[0064]

In this embodiment, the metal catalyst can be accommodated in the hydrophobic pocket of HSA by linking the metal catalyst with a ligand against HSA, and then binding the metal catalyst within the hydrophobic pocket via said ligand. The ligand that can be used for this purpose may change depending on the hydrophobic pocket that accommodates the metal catalyst. For example, when accommodating the metal catalyst in the albumin drug binding site I of HSA, said ligand can be selected from warfarin, azapropazone, acenocoumarol, phenylbutazone, salicylate salt, indomethacin, phenytoin, tolbutamide, chlorpropamide, iophenoxate, iodipamide, sulfadimethoxine, phenprocoumon, glibenclamide, sulfathiazole, tenoxicam, camptothecin, balzidendeazi, andelleratan, diadelleal, diadellealtan, prodan, bilirubin, eicosanoid, and carboxy-methyl-propyl-furanpropanoate (uremic toxin), as well as coumarin. Alternatively, when accommodating the metal catalyst in the albumin drug binding site II of HSA, said ligand can be selected from diazepam, ketoprofen, chlofibrate, ibuprofen, iopanoate, azide deoxythymidine, flufenamate, ethacrynate, naproxen, flurbiprofen, cicloprofen, benoxaprofen, flucloxacillin, chlorothiazide, piroprofen, propofol, isoflurane, dansylsarcosine, dansylglycine, alkylaminocoumarin acetic acid, hydroxyflavone, L-tryptophan, medium chain fatty acid anion (such as octanoate), L-thyroxine, chloride ion, iodoacetic acid, indoxyl sulfate, and hippuric acid (urotoxin). In one embodiment of the present invention, the hydrophobic pocket of HSA is the drug binding site I, and a coumarin derivative, e.g. 7-dimethylamino coumarin is used as the ligand in order to accommodate ruthenium in the aforementioned site.

[0065]

The linking between the metal catalyst and said ligand may be performed via a linker. As the linker that can be used for this purpose, an alkyl chain or a polyethylene glycol (PEG) chain etc. having an amino group and a carboxy group on both ends can be

typically employed. Specifically, alkyl chain linkers can include, for example, $\text{-NH-(CH}_2\text{)}_x\text{-CO-}$ (wherein x is an integer and is not limited as long as it does not inhibit the target linker function, and for example can be an integer between 1 - 15), $\text{-NH-(CH}_2\text{CH}_2\text{O)}_y\text{-CH}_2\text{-CO-}$ (wherein y is an integer and is not limited as long as it does not inhibit the target linker function, and for example can be an integer between 1 - 15), or $\text{-NH-(CH}_2\text{CH}_2\text{O)}_z\text{-CH}_2\text{CH}_2\text{-CO-}$ (wherein z is an integer and is not limited as long as it does not inhibit the target linker function, and for example can be an integer between 1 - 15), and the like.

[0066]

When a linker is employed for linking the metal catalyst and said ligand, it should be noted that the length of the linker used is a length such that the metal catalyst is accommodated within the hydrophobic pocket of HSA and is not exposed or not substantially exposed to the hydrophilic environment. For example, when accommodating ruthenium in the albumin drug binding site I of HSA employing coumarin or a derivative thereof (such as 7-dimethylamino coumarin, 7-diethylamino coumarin (DEAC)) as the ligand, "not substantially exposed to the hydrophilic environment" may be that the area of ruthenium exposed to the hydrophilic environment is 40% or less, and preferably 35% or less of the total surface area of ruthenium. Alternatively, "not substantially exposed to the hydrophilic environment" may be that the SASA of ruthenium when accommodated in the albumin drug binding site I of HSA is 3.0 or less, and preferably 1.0 or less. Moreover, an exemplary linker used in this case is $\text{-NH-(CH}_2\text{CH}_2\text{O)}_y\text{-CH}_2\text{-CO-}$, wherein y may be an integer from 1 to 6, and preferably an integer from 1 to 3.

[0067]

In one embodiment, in the ArM of the present invention which is a complex of HSA and a metal catalyst, the surface of HSA is modified with a sugar chain. The content of the glycosylation may change depending on the *in vivo* target. The modification position on the HSA surface is not particularly limited as long as

introduction of a sugar chain is possible, and an example can include the lysine residue at position 30 on the HSA surface.

[0068]

The complex of the present invention can activate a prodrug *in vivo* depending on the type of the catalyst accommodated in the aforementioned complex. Preferably, the complex of the present invention, depending on the type of the catalyst accommodated in the aforementioned complex, is accumulated at a particular position *in vivo* to allow activation of the prodrug selective to the aforementioned position.

[0069]

Accordingly, in another aspect, the present invention relates to a composition comprising the complex of the present invention.

[0070]

In one embodiment, the composition of the present invention is a pharmaceutical composition that is used in combination with a prodrug that can be activated by the complex of the present invention. In the aforementioned embodiment, the pharmaceutical composition of the present invention may be a single dosage form further comprising a pharmaceutically acceptable carrier, and depending on the case a prodrug that can be activated by the complex of the present invention. Prodrugs that can be activated by the complex of the present invention that can be used in the present invention can include e.g. various anticancer agents, and specifically, but is not limited thereto, mitomycin C, doxorubicin, taxol, endoxifen, and the like can be exemplified.

[0071]

In another embodiment, the pharmaceutical composition of the present invention is in a form of a combination medicine wherein the complex of the present invention and a prodrug that can be activated by the aforementioned complex are provided as separate agents. In the combination medicine of the present invention, a first agent comprising the complex of the present invention and a second agent comprising a prodrug that can be

activated by the aforementioned complex can be administered to the subject at same or different times.

[0072]

In another aspect, the composition of the present invention relates to a pharmaceutical composition comprising a prodrug that can be activated by the complex of the present invention and any pharmaceutically acceptable carrier, and the aforementioned pharmaceutical composition can be characterized by being used in combination with the complex of the present invention.

[0073]

In one embodiment, the pharmaceutical composition of the present invention can be employed for selectively tagging particular cells *in vivo*. In the present invention, "tagging" refers to employing a non-toxic chemical substance that may be intrinsic or extrinsically administered and can destroy cell function (such as an adherence inhibitor) or can elicit immunological response, in order to tag a target cell. Such a chemical substance may be an *in vivo* or *ex vivo* chemical substance that is converted from an inactive form to an active form by any metal catalyst, or an *in vivo* or *ex vivo* chemical substance that is enhanced in its function by any metal catalyst. Such a method in therapeutic application is termed selective cell tagging (SeCT), and in contrast to conventional chemotherapy that directly removes cancer cells with a highly cytotoxic agent, it is expected to indirectly induce target cell (such as cancer cell) death without significantly damaging the surrounding tissue. For example, in the Examples herein, a possibility is shown that the binding of cRGD-propargyl ester (cRGD-PE) against integrins expressed on the cancer cell surface is enhanced by the complex of the present invention, and metastasis can be inhibited.

[0074]

In another embodiment, the composition of the present invention can be employed as a biosensor. Specifically, in the complex of the present invention, by employing a metal catalyst, a linker having fluorescence per se and/or having fluorescence that is enhanced by binding with proteins such as albumin (such as a

coumarin derivative), and a quencher that may depart by a reaction between the metal catalyst and the detection target substance, the complex or composition of the present invention can be designed as an appropriate biosensor according to the type of the detection target substance. In a specific embodiment of the present invention, the composition of the present invention is employed as a biosensor for detecting ethylene in plants.

[0075]

Note that the terms used herein are to be employed to describe particular embodiments, and do not intend to limit the invention.

[0076]

Moreover, the term "comprising" as used herein, unless the content clearly indicates to be understood otherwise, intends the presence of the described items (such as components, steps, elements, and numbers), and does not exclude the presence of other items (such as components, steps, elements, and numbers).

[0077]

Unless otherwise defined, all terms used herein (including technical and scientific terms) have the same meanings as those broadly recognized by those skilled in the art of the technology to which the present invention belongs. The terms used herein, unless explicitly defined otherwise, should be construed as having meanings consistent with the meanings herein and in related technical fields, and shall not be construed as having idealized or excessively formal meanings.

[0078]

Although terms such as first and second may be employed to express various elements, it should be recognized that these elements are not to be limited by these terms. These terms are employed solely for the purpose of discriminating one element from another, and for example, it is possible to describe the first element as the second element, and similarly to describe the first element as the second element without departing from the scope of the present invention.

[0079]

The present invention will now be more specifically described by Examples. However, the present invention can be embodied by various embodiments, and shall not be construed as being limited to the Examples described herein.

Examples

[0080]

[Example 1]

Manufacture of alb-Ru

In order to design a biocompatible artificial metal enzyme (ArM) that can prevent the exposure of the metal catalyst into the solvent (has a binding pocket), the drug binding site I (drug site I) of albumin (hydrophobic binding pocket as the pseudo-active site) was utilized. In order to accomplish the objective, a metal catalyst was fixed to the drug binding site I known for the binding (interaction) of coumarin derivatives (such as warfarin) (Ghuman, J.; Zunszain, P. A.; Petitpas, I.; Bhattacharya, A. A.; Otagiri, M.; Curry, S. J. Mol. Biol. 2005, 353, 38-52). Coumarin-Ru complexes Ru1 - 3, 6 having differing PEG linker lengths was employed for fixing the metal catalyst.

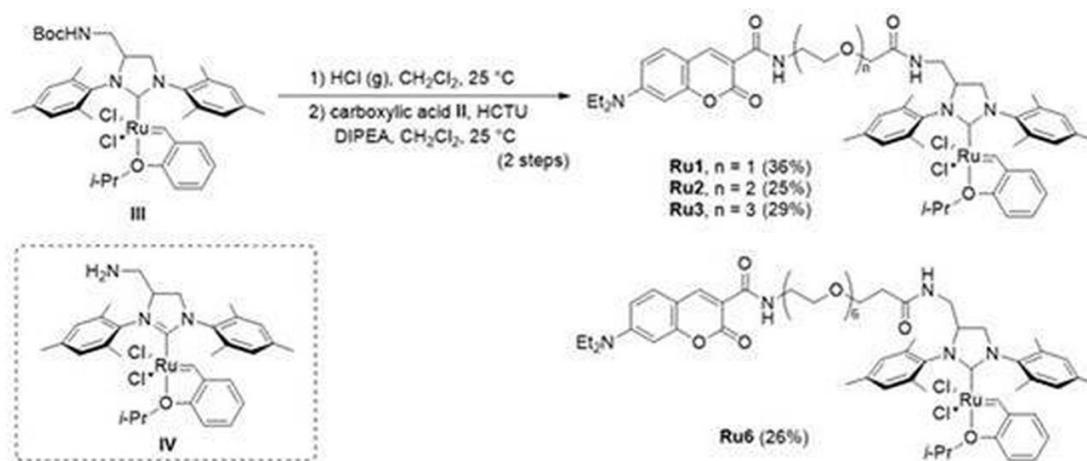
[0081]

1. Preparation of coumarin-Ru complexes

1-1. General procedure B

According to the following reaction scheme, coumarin-bound ruthenium complexes Ru1 - 3, 6 can be synthesized.

[Chemical Formula 1]



[0082]

Ruthenium complex III is prepared according to a well-known technology (Lo, C.; Ringenberg, M. R.; Gndt, D.; Wilson, Y.; Ward, T. R. *ChemComm* 2011, 47, 12065-12067; Kajetanowicz, A.; Chatterjee, A.; Reuter, R.; Ward, T. R. *Catal. Lett.* 2014, 144, 373-379; Zhao, J.; Kajetanowicz, A.; Ward, T. R. *Org. Biomol. Chem.* 2015, 13, 5652-5655). To a solution of ruthenium complex III (80.0 mg, 0.106 mmol) in dichloromethane (3 mL), hydrochloric acid gas is bubbled at 25°C. Hydrochloric acid gas is prepared by adding concentrated sulfuric acid dropwise to ammonium chloride. After stirring for 45 minutes, dichloromethane (1 mL) is added to the reactant with a syringe, and this is stirred at the same temperature. After another 15 minutes, the reactant is concentrated under reduced pressure to obtain amine IV, and this is used for the next reaction without purification.

[0083]

In another flask, a solution of carboxylic acid IIa (1.1 equivalents) and a coupling agent HCTU (1.3 equivalents) dissolved in dichloromethane (1 mL) is stirred at 25°C for 30 minutes. To this reactant, amine IV (1 equivalent) dissolved in dichloromethane (1 mL) was added, followed by N,N-diisopropylethylamine (10 equivalents) at the same temperature. After stirring for 6 hours, the reaction is stopped by adding 1 M HCl aqueous solution. The product is extracted three times with dichloromethane, the combined organic extract layer is washed with saturated sodium bicarbonate water and brine, dried (by sodium sulfate), and then concentrated under reduced pressure. The concentrated residue is purified by silica gel flash column chromatography, and coumarin-Ru complexes Ru1 - 3, 6 can be obtained.

[0084]

1-2. Preparation of coumarin-Ru complex Ru1

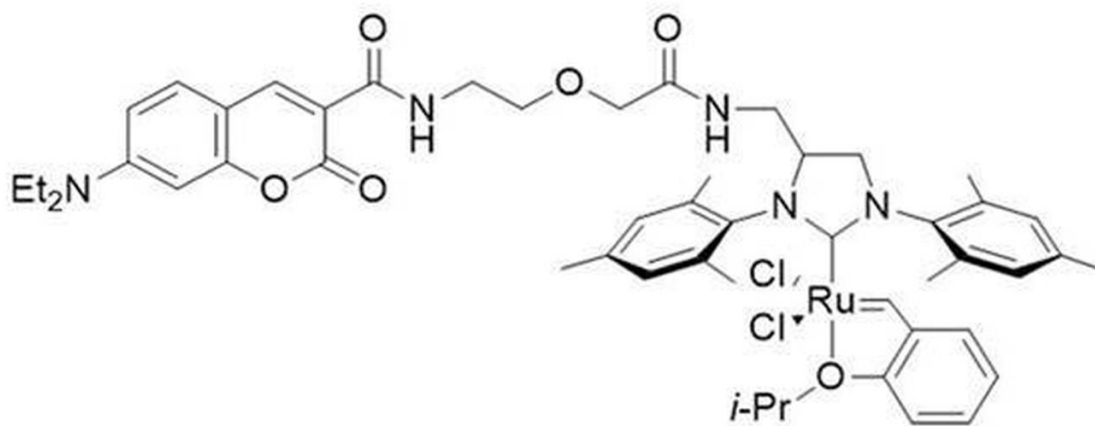
According to general procedure B, after purification of the reactant obtained from ruthenium complex III (80.1 mg, 0.106 mmol), carboxylic acid IIa (42.4 mg, 0.117 mmol), HCTU (57.6 mg, 0.139

mmol), and *N,N*-diisopropylethylamine (137 mg, 1.06 mmol) by silica gel flash column chromatography (cyclohexane/EtOAc/CHCl₃/MeOH = 40/40/15/5), the target coumarin-Ru complex Ru1 (37.7 mg, 35.6%) is obtained as a green solid.

¹H-NMR (400 MHz, CDCl₃, δ) 1.24–1.27 (m, 12H), 2.37–2.51 (br m, 18H), 3.47 (q, 4H, *J* = 7.1 Hz), 3.53–3.71 (m, 6H), 3.79 (m, 1H), 3.93 (d, 1H, *J* = 15.3 Hz), 3.99 (d, 1H, *J* = 15.3 Hz), 4.05 (dd, 1H, *J*₁ = *J*₂ = 10.0 Hz), 4.33 (dd, 1H, *J*₁ = *J*₂ = 10.0 Hz), 4.75–4.84 (br m, 1H), 4.89 (sept, 1H, *J* = 6.1 Hz), 6.50 (d, 1H, *J* = 2.3 Hz), 6.66 (dd, 1H, *J*₁ = 2.3 Hz, *J*₂ = 9.1 Hz), 6.78 (d, 1H, *J* = 7.7 Hz), 6.85 (dd, 1H, *J*₁ = *J*₂ = 7.7 Hz), 6.91 (dd, 1H, *J*₁ = 1.7 Hz, *J*₂ = 7.7 Hz), 7.02 (s, overlapped, 2H), 7.04 (s, 1H), 7.07 (s, 1H), 7.42 (d, 1H, *J* = 9.1 Hz), 7.48 (ddd, 1H, *J*₁ = 1.7 Hz, *J*₂ = *J*₃ = 7.7 Hz), 8.67 (s, 1H), 9.09 (s, 1H), 16.50 (s, 1H);

HRMS (ESI) *m/z* 964.3355 (964.3359 calcd for C₅₀H₆₁ClN₅O₆Ru, [M-Cl]⁺).

[Chemical Formula 2]



Ru1

[0085]

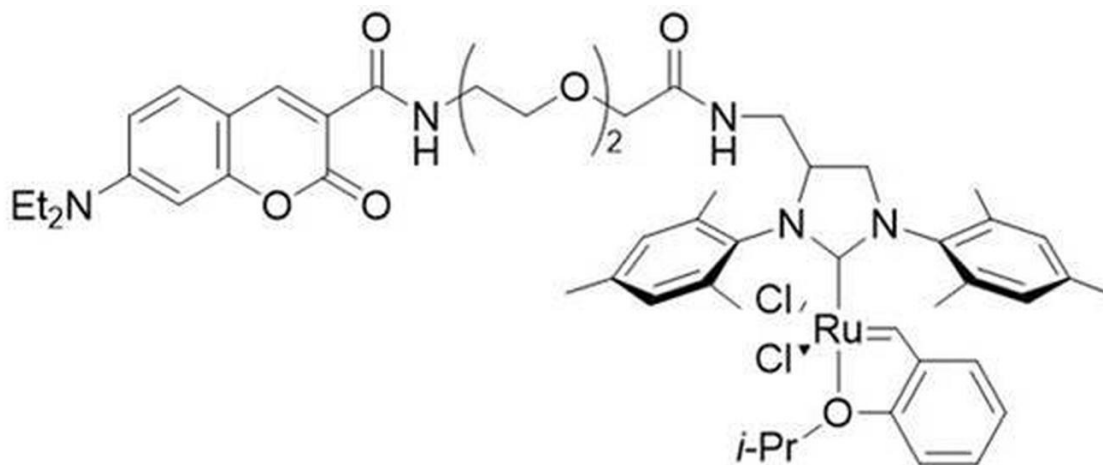
1-3. Preparation of coumarin-Ru complex Ru2

According to general procedure B, after purification of the reactant obtained from ruthenium complex III (80.1 mg, 0.106 mmol), carboxylic acid IIb (47.4 mg, 0.117 mmol), HCTU (57.1 mg, 0.138 mmol), and *N,N*-diisopropylethylamine (137 mg, 1.06 mmol) by silica gel flash column chromatography (cyclohexane/EtOAc/CHCl₃/MeOH = 40/40/15/5), the target coumarin-Ru complex Ru2 (27.9 mg, 25.2%) was obtained as a green solid.

$^1\text{H-NMR}$ (400 MHz, CDCl_3 , δ) 1.22-1.26 (m, 12H), 2.26-2.46 (br m, 18H), 3.38-3.54 (m, 4H), 3.61-3.70 (m, 10H), 3.74-3.82 (m, 1H), 3.97 (s, 2H), 4.11 (dd, 1H, $J_1 = J_2 = 10.3$ Hz), 4.28 (dd, 1H, $J_1 = J_2 = 10.3$ Hz), 4.66-4.75 (br m, 1H), 4.88 (sept, 1H, $J = 6.1$ Hz), 6.47 (d, 1H, $J = 2.3$ Hz), 6.65 (dd, 1H, $J_1 = 2.3$ Hz, $J_2 = 9.0$ Hz), 6.77 (d, 1H, $J = 7.6$ Hz), 6.83 (dd, 1H, $J_1 = J_2 = 7.6$ Hz), 6.89 (dd, 1H, $J_1 = 1.9$ Hz, $J_2 = 7.6$ Hz), 6.98-7.01 (br m, 4H), 7.43 (d, 1H, $J = 9.0$ Hz), 7.47 (ddd, 1H, $J_1 = 1.9$ Hz, $J_2 = J_3 = 7.6$ Hz), 8.67 (s, 1H), 9.05 (s, 1H), 16.47 (s, 1H);

HRMS (ESI) m/z 1008.3613 (1008.3622 calcd for $\text{C}_{52}\text{H}_{65}\text{ClN}_5\text{O}_7\text{Ru}$, $[\text{M}-\text{Cl}]^+$).

[Chemical Formula 3]



Ru2

[0086]

1-4. Preparation of coumarin-Ru complex Ru3

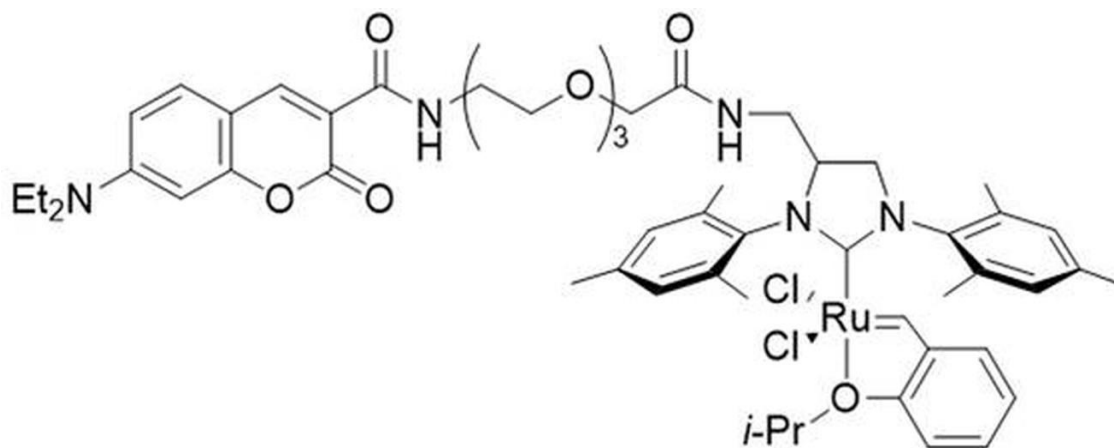
According to general procedure B, after purification of the reactant obtained from ruthenium complex III (28.8 mg, 38.1 μmol), carboxylic acid IIc (18.9 mg, 42.0 μmol), HCTU (21.3 mg, 51.5 μmol), *N,N*-diisopropylethylamine (49.7 μg , 385 μmol) by silica gel flash column chromatography (cyclohexane/EtOAc/ CHCl_3 /MeOH = 40/40/15/5), the target coumarin-Ru complex Ru3 (12.0 mg, 28.9%) was obtained as a green solid.

$^1\text{H-NMR}$ (400 MHz, CDCl_3 , δ) 1.22-1.28 (m, 12H), 2.34-2.44 (br m, 18H), 3.46 (q, 4H, $J = 7.2$ Hz), 3.55-3.70 (m, 14H), 3.71-3.80 (m, 1H), 3.95 (s, 2H), 4.04 (dd, 1H, $J_1 = J_2 = 10.7$ Hz), 4.27 (dd, 1H, $J_1 = J_2 = 10.7$ Hz), 4.57-4.65 (m, 1H), 4.89 (sept, 1H, $J = 6.2$ Hz),

6.47 (d, 1H, $J = 2.3$ Hz), 6.65 (dd, 1H, $J_1 = 2.3$ Hz, $J_2 = 8.8$ Hz), 6.79 (d, 1H, $J = 7.7$ Hz), 6.84 (dd, 1H, $J_1 = J_2 = 7.6$ Hz), 6.89 (dd, 1H, $J_1 = 1.9$ Hz, $J_2 = 7.6$ Hz), 7.01-7.05 (br m, 4H), 7.42 (d, 1H, $J = 8.8$ Hz), 7.47 (ddd, 1H, $J_1 = 1.9$ Hz, $J_2 = J_3 = 7.6$ Hz), 8.67 (s, 1H), 8.98 (s, 1H), 16.47 (s, 1H);

HRMS (ESI) m/z 1088.3661 (1088.3648 calcd for $C_{54}H_{70}Cl_2N_5O_8Ru$, $[M+H]^+$).

[Chemical Formula 4]



Ru3

[0087]

1-5. Preparation of coumarin-Ru complex Ru6

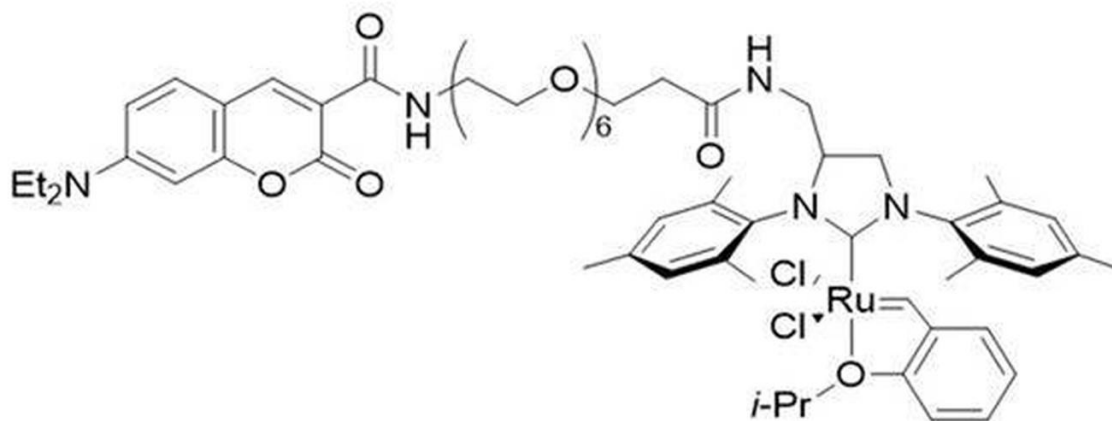
According to general procedure B, after purification of ruthenium complex III (80.4 mg, 0.106 mmol), carboxylic acid IIId (69.9 mg, 0.117 mmol), HCTU (57.5 mg, 0.139 mmol), *N,N*-diisopropylethylamine (137 mg, 1.06 mmol) by silica gel flash column chromatography (cyclohexane/EtOAc/ $CHCl_3$ /MeOH = 20/20/55/5), the target coumarin-Ru complex Ru6 (34.4 mg, 26.3%) was obtained as a green solid.

1H -NMR (400 MHz, $CDCl_3$, δ) 1.21-1.30 (m, 12H), 2.37-2.45 (br m, 18H), 3.45 (q, 4H, $J = 7.3$ Hz), 3.56-3.68 (m, 31H), 3.99 (dd, 1H, $J_1 = J_2 = 10.5$ Hz), 4.25 (dd, 1H, $J_1 = J_2 = 10.5$ Hz), 4.53-4.62 (br m, 1H), 4.91 (sept, 1H, $J = 6.1$ Hz), 6.49 (d, 1H, $J = 2.3$ Hz), 6.64 (dd, 1H, $J_1 = 2.3$ Hz, $J_2 = 8.8$ Hz), 6.80 (d, 1H, $J = 7.6$ Hz), 6.86 (dd, 1H, $J = 7.6$ Hz), 6.90 (dd, 1H, $J_1 = 1.9$ Hz, $J_2 = 7.6$ Hz), 7.03-7.07 (br m, 4H), 7.42 (d, 1H, $J = 8.8$ Hz), 7.48 (ddd, 1H, J_1

= 1.9 Hz, $J_2 = J_3 = 7.6$ Hz), 8.68 (s, 1H), 9.00 (s, 1H), 16.47 (s, 1H);

HRMS (ESI) m/z 1234.4603 (1234.4593 calcd for $C_{61}H_{84}Cl_2N_5O_{11}Ru$, $[M+H]^+$).

[Chemical Formula 5]



Ru6

[0088]

2. Manufacture of alb-Ru

The manufacture of alb-Ru was performed by reacting ruthenium catalysts Ru1 - 3, 6 and human serum albumin (HAS) to form alb-Ru complexes.

The composition of the reaction solution contained 30 μ M human serum albumin (hereinafter HSA; 167 μ L of 50 nmol, 300 μ M stock solution (aqueous solution) used) and the catalyst (Ru1 - 3, 6) at various concentrations. The catalyst used was for example 37 μ M of Ru1 (167 μ L of 62 nmol, 370 μ M stock solution (dioxane solution) used). The total reaction volume was filled to 1670 μ L with PBS buffer (pH 7.4) comprising 10% dioxane. After starting the reaction by addition of HSA, the reaction mixture was gently mixed and incubated at 37°C for 1 hour. Subsequently, with Amicon™ ultracentrifugation filter (30 kDa), the reaction solution was washed with PBS buffer and concentrated. Next, the concentrated alb-Ru solution was diluted with PBS buffer, and 1000 μ L was obtained as 50 μ M stock solution.

[0089]

For confirmation of Alb-Ru complex formation, fluorescence assay that depends on the fact that coumarin-based molecules are sensitive to the polarity of the solvent was employed. Using Ru1 - 3, 6, the fluorescence intensity (measured value) obtained from 1:1 reaction of ligand-albumin is shown in Figure 1.

As compared to the control (ligand only or HSA only), significantly high fluorescence level that is thought to be the indicator of alb-Ru complex formation was detected.

[0090]

Moreover, in order to indirectly determine the Ru1 - 3, 6 binding site, HSA solution was preincubated together with 2 equivalents of either of the binding ligand (warfarin or ibuprofen) at 37°C for 1 hour. Subsequently, the saturated binding curve of Ru1 - 3, 6 was generated with a mixture of albumin and binding ligand (Figure 2).

[0091]

From the results of Figure 2, it became clear that the binding of Ru1 - 3, 6 was unaffected in the presence of ibuprofen, but significantly decreased in the presence of warfarin. This result strongly suggests that the drug binding site I of HSA is the main binding site of these compounds.

[0092]

[Example 2]

Verification of reactivity of alb-Ru

Next, the ability of the alb-Ru complex to catalyze ring-closing metathesis (RCM) of olefin 1a - 1d and enyne 1e was examined (Figure 3). Moreover, since alb-Ru1 - 3, 6 has differing PEG linker lengths, effective size and compatibility of the hydrophobic binding pocket was also verified.

[0093]

The reaction solution essentially contained 1a - e as the substrate and the alb-Ru complex (i.e. Ru1 - 3, 6) in 1:9 dioxane:PBS buffer (pH 7.4). The reaction, after incubation at 37°C for 2 hours, was quenched with dodecane thiol, further diluted with methanol, and the sample was filtered, subjected to

HPLC analysis, and the metabolic turnover (TON) into respective products 2a - e was calculated. As control, a reaction using free ruthenium catalyst Ru1 - 3, 6 in solution (free-in-solution) was also carried out under similar conditions.

[Table 1]

Entry	Sub.	Catalyst	Catalyst Linker Length				
			Ru1 (n = 1)		Ru2 (n = 2)	Ru3 (n = 3)	Ru6 (n = 6)
			TON	r	TON	TON	TON
1	1a	alb-Ru	0.9 ± 0.01	0.35	0.3 ± 0.01	0.1 ± 0.01	0.2 ± 0.01
2		Ru	2.6 ± 0.05		1.5 ± 0.04	1.3 ± 0.01	0.8 ± 0.10
3	1b	alb-Ru	2.3 ± 0.06	0.34	0.8 ± 0.01	0.3 ± 0.01	0.5 ± 0.01
4		Ru	6.9 ± 0.01		6.3 ± 0.02	5.2 ± 0.01	5.5 ± 0.03
5	1c	alb-Ru	0.4 ± 0.19	0.25	0.2 ± 0.02	0.1 ± 0.01	0.1 ± 0.01
6		Ru	1.6 ± 0.02		1.9 ± 0.02	1.5 ± 0.10	1.8 ± 0.36
7	1d	alb-Ru	19.7 ± 0.17	0.59	2.8 ± 0.04	0.9 ± 0.01	0.5 ± 0.03
8		Ru	33.2 ± 0.18		28.8 ± 3.77	28.8 ± 0.17	34.7 ± 0.18
9	1e	alb-Ru	29.9 ± 0.20	0.57	1.2 ± 0.05	1.3 ± 0.01	0.6 ± 0.03
10		Ru	52.5 ± 0.10		55.7 ± 0.34	58.6 ± 0.10	41.5 ± 0.19

[0094]

One of the first observations in this experiment was the correlation between the increase in the length of the PEG linker (between coumarin anchor and ruthenium catalyst) and the decrease in activity. For example, the metabolic turnover of the substrate 4,4-bis((benzoyloxy)methyl)-1,6-heptadiene 1d (Entry 7) was the highest in the presence of alb-Ru1 (19.7), abruptly declined in the presence of alb-Ru2 (2.8), and showed minuscule levels in the presence of alb-Ru6 (0.5). Given that the metabolic turnover (TON) obtained using free ruthenium catalyst Ru1 in solution generally remains within the range of 28 - 35 for all of the linker lengths employed (Entry 8), this observation result suggests that the hydrophobic binding pocket cannot accommodate a coumarin anchor-catalyst complex having a long linker.

[0095]

[Example 3]

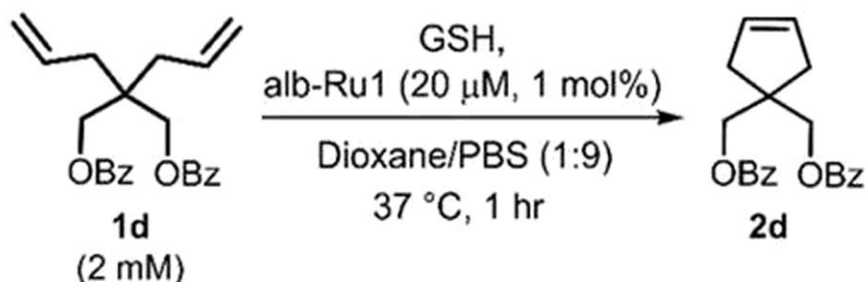
Verification of biocompatibility of alb-Ru

After confirming that the alb-Ru complex shows activity to catalyze ring-closing metathesis and enyne cross metathesis, we then aimed for verification of biocompatibility by evaluating the action of glutathione. Compound 1d was selected as the model substrate for this research.

[0096]

One of the important experiments in this research was to examine the catalyst protection ability of alb-Ru1 against glutathione. As shown below, 1 mol% alb-Ru1 was employed, and substrate 1d was reacted together with addition of various concentrations of GSH. Note that depending on the concentration of alb-Ru1, glutathione was added at equal equivalents.

[Chemical Formula 6]



[0097]

As a result, change in (calculated) TON was not observed up to 20 mM GSH (1000 equivalents of GSH against alb-Ru). Ultimately, with addition of 100 mM and 200 mM GSH, 60% and 82% decrease in TON compared to control was respectively recognized.

[0098]

In the next investigation, in order to learn the upper threshold limit of the GSH protection ability, the duration of the metal catalyst protection was evaluated. Specifically, alb-Ru1 was preincubated together with any of GSH, dodecane thiol, or PBS buffer (as blank) for various hours, then substrate 1d was added to the reaction solution, and the TON value was calculated after 1 hour of reaction. Figure 4 shows the experiment of performing with physiologically appropriate GSH concentration (200 μ M, 10 equivalents against alb-Ru1).

[0099]

The TON value measured after 6 hours of preincubation with GSH generally only showed slight decrease compared to the blank (PBS buffer). In contrast, in the experiment employing GSH at the upper threshold limit (20 mM, 1000 equivalents against alb-Ru1), a prominent (much larger) effect on reactivity was shown (Figure 5).

[0100]

In such a case, about 50% decrease in activity was observed after 1 hour of preincubation with GSH, whereas the activity nearly disappeared after 4 hours of preincubation.

[0101]

In order to further evaluate the biocompatibility of Alb-Ru complex, substrate 1d was incubated with 10 mol% alb-Ru1 complex at 37°C for 2 hours. The production yield of 2d was about 2% with 1:8:1 fetal bovine serum/DMEM medium/dioxane, and about 1% with 1:8:1 normal rat serum/PBS buffer/dioxane. However, since the collected starting material 1d was 8% and 3% respectively, the low production yield is likely to be attributable to the capture or degradation of the substrate by proteins that commonly exist in the serum.

[0102]

In regards to ArM activity (artificial metal enzyme activity), Michaelis-Menten kinetic parameters of substrates 1a - e were calculated with or without addition of GSH (Figure 6).

[0103]

As a result, regardless of with or without GSH addition, there was only a slight change (within error) in kinetic numeric values measured for 1a-1e in ArM activity. Nonetheless, of particular importance is the difference in catalyst efficiency (k_{cat}/K_M) observed between these substrates. In particular, substrate 1e shows a k_{cat}/K_M value of about $3 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, and although this value compares poorly with the reactivity of a natural enzyme ($k_{\text{cat}}/K_M - 10^8 \text{ M}^{-1}\text{s}^{-1}$), it is emphasized from this result that there is a possibility that the ArM of non-natural metal will reach reactivity equivalent to a natural enzyme.

[0104]

[Example 4]

Mechanism of glutathione resistance

In order to predict the extent of invasion and conformation in the hydrophobic binding pocket of the drug binding site I of albumin, Autodock 4.2 (Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. J.

Comput. Chem. 2009, 30, 2785-2791.) was employed in the GUI interface of AutoDockTools to research compound Ru1 - 3, 6 for molecular modeling that docks with human serum albumin (PDB:1H9Z). The result supports the fundamental assumption that the binding pocket of the drug binding site I of albumin is deep enough to accommodate the binding of a coumarin-ruthenium catalyst (e.g. Ru1) having a relatively short PEG linker length. However, as shown in Figure 7, as the PEG linker length gradually gets longer (e.g. Ru6), the ruthenium portion gets pushed to the outside of the binding pocket, and its exposure to biomolecules in the solution is increased.

[0105]

This theory was further supported by calculation of the relative solvent accessible surface area (SASA) of the ruthenium atom of the docking ligand, with the fact that the correlation with a longer PEG linker is increased. Note that in Figure 7, zero value indicates atoms not exposed to the solvent.

[0106]

[Example 5]

Targeting of artificial metal enzyme

Another aspect to be considered for promoting the development of therapeutic ArM is the need for a targeting methodology to facilitate localization to particular organs/cells in the body.

[0107]

If successful, application to prodrug therapy will be possible, which in turn is particularly beneficial for the development of medicinal candidates having risks of side effects, such as anti-cancer therapy based on cytotoxic molecules.

[0108]

It has been found that since the complexity of glycocalyx that envelops the surface of different eucaryotic cells is changing, glycoalbumin bound to a particular combined N-glycan conglomerate can exert different recognition ability or binding between different cancer cells. In an essential research with the

objective to test whether these glycoalbumins have the ability to act as *in vivo* supports, in regards to protein labeling based on propargylic ester, glycoalbumin comprising a gold catalyst was localized in a particular organ of a live mouse by the N-glycan structure thereof and showed *in vivo* catalytic activity (Tsubokura, K.; Vong, K. K. H.; Pradipta, A. R.; Ogura, A.; Urano, S.; Tahara, T.; Nozaki, S.; Onoe, H.; Nakao, Y.; Sibgatullina, R.; Kurbangalieva, A.; Watanabe, Y.; Tanaka, K. *Angew. Chem. Int. Ed. Engl.* 2017, 56, 3579-3584.).

[0109]

In order to develop and investigate applicability to cancer, a uniform conglomerate of N-glycans at the sialic acid $\alpha(2,3)$ -linked terminal was selected as the target group. This selection was made based on a previous research which showed that glycoalbumin(2,3-Sia) having sialic acid $\alpha(2,3)$ -bond can preferentially accumulate in SW620 human colon adenocarcinoma cells, which also showed moderate binding to A549 human pulmonary alveolar basement epithelial adenocarcinoma cells and HeLa human cervical cancer-derived cells (Ogura, A.; Urano, S.; Tahara, T.; Nozaki, S.; Sibgatullina, R.; Vong, K.; Suzuki, T.; Dohmae, N.; Kurbangalieva, A.; Watanabe, Y.; Tanaka, K. *ChemComm* 2018, 54, 8693-8696.). This effect is likely caused by the overexpression of galectin-8 which is known as a well-known $\alpha(2,3)$ -linked sialic acid (Lahm, H.; Andre, S.; Hoeflich, A.; Fischer, J. R.; Sordat, B.; Kaltner, H.; Wolf, E.; Gabius, H.-J. *J. Cancer Res. Clin. Oncol.* 2001, 127, 375-386; Carlsson, S.; Oberg, C. T.; Carlsson, M. C.; Sundin, A.; Nilsson, U. J.; Smith, D.; Cummings, R. D.; Almkvist, J.; Karlsson, A.; Leffler, H. *Glycobiology* 2007, 17, 663-676.).

[0110]

First, glycosylated ArM (GArM)-Ru1(2,3-Sia) having ruthenium catalyst Ru1 fixed thereon was prepared, and then the targeting ability towards SW620 human colon adenocarcinoma cells was evaluated in a binding experiment. In order to evaluate specific binding of GArM-Ru1(2,3-Sia) to cancer cells, cancer cells were seeded in a 96-well plate with a transparent bottom at

a density of 10^4 cells/well, and cultured overnight. Subsequently, the medium was removed, and [1] GArM-Ru1(2,3-Sia), [2] alb-Ru, [3] GA(2,3-Sia), or [4] Ru1 were added so that each will have a final concentration of 10 μ M. After incubating the cells at 37°C for 3 hours, the medium was removed, this was washed with PBS buffer (3 times), and fixed on the plate with formaldehyde reagent. Cell observation was performed with BZ-X710 All-in-one Fluorescence Microscope™ from Keyence, and fluorescent images and bright field images were recorded. ET-EBFP2/Coumarin/Attenuated DAPI Filter Set Cat# 49021 (Chroma Technology Corp., Vermont, USA) was employed for detection of coumarin-derived fluorescence. Imaging images were taken at 20 X magnification, and analyzed with BZ-X Analyzer (from Keyence) software.

[0111]

The result is shown in Figure 8. By cell imaging utilizing the fluorescent luminescence of coumarin bound to HSA, a more intense fluorescence accumulation was recognized in SW620 human colon adenocarcinoma cells incubated with GArM-Ru1(2,3-Sia) compared to its control.

[0112]

As an additional test, a similar experiment was also performed with HeLa human cervical cancer-derived cells and A549 human pulmonary alveolar basement epithelial adenocarcinoma cells. Consistent with previous reports, it was observed that accumulation of GArM-Ru1(2,3-Sia) in SW620 human colon adenocarcinoma cells was higher than that in HeLa human cervical cancer-derived cells and A549 human pulmonary alveolar basement epithelial adenocarcinoma cells (Figure 9).

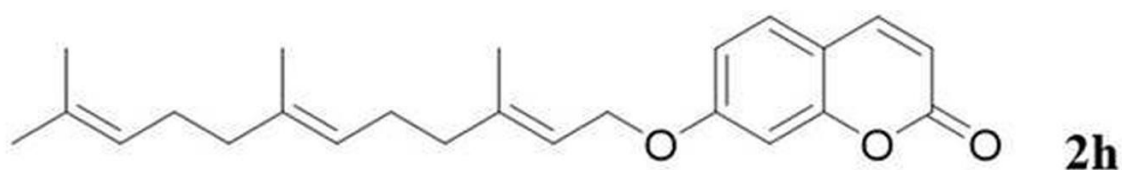
[0113]

[Example 6]

Prodrug therapy

Next, applicability of GArM-Ru complex targeting anticancer therapy was investigated. Umbelliprenin (2h) was selected as the initial investigation of drug candidates that are activated by ring-closing metathesis.

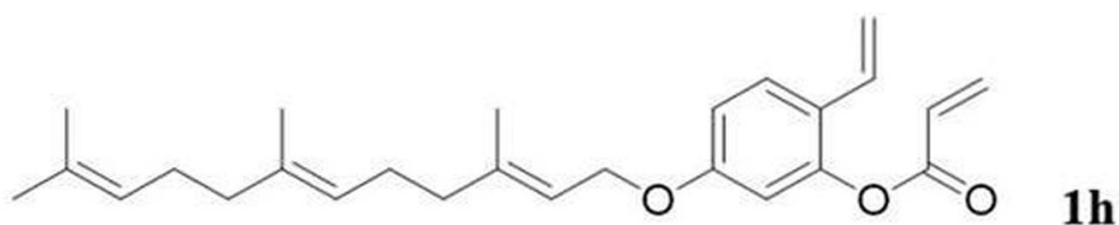
[Chemical Formula 7]



[0114]

Umbelliprenin which is known as a natural product extracted from *Ferula* plants has shown cytotoxic activity against various cancer cell strains (Shakeri, A.; Iranshahy, M.; Iranshahi, M. J. Asian. Nat. Prod. Res. 2014, 16, 884-889; Rashidi, M.; Khalilnezhad, A.; Amani, D.; Jamshidi, H.; Muhammadnejad, A.; Bazi, A.; Ziai, S. A. J. Cell. Physiol. 2018, 233, 8908-8918; Jun, M.; Bacay, A. F.; Moyer, J.; Webb, A.; Carrico-Moniz, D. Bioorganic Med. Chem. Lett. 2014, 24, 4654-4658). The mechanism of the cytotoxicity is shown to be induction of apoptosis by arresting of the cell in G1 phase, further activation of caspase-8 and 9, and down regulation of Bcl-2 and Mcl-1 (Barthomeuf, C.; Lim, S.; Iranshahi, M.; Chollet, P. Phytomedicine 2008, 15, 103-111; Gholami, O.; Jeddi-Tehrani, M.; Iranshahi, M.; Zarnani, A. H.; Ziai, S. A. Iran J. Pharm. Res. 2013, 12, 371-376; Gholami, O.; Jeddi-Tehrani, M.; Iranshahi, M.; Zarnani, A. H.; Ziai, S. A. Iran J. Pharm. Res. 2014, 13, 1387-1392). Due to its very high hydrophobicity (mainly rendered by the farnesyl portion), umbelliprenin prodrug (1h) was theorized to be an ideal substrate for albumin-based ArM.

[Chemical Formula 8]



[0115]

In order to verify this, simplified coumarin derivative 1g and umbelliprenin prodrug 1h were both employed for kinetic experiment (Figure 10).

[0116]

Although coumarin precursors are ordinarily known to have poor RCM reactivity under aqueous conditions, a very slight reactivity ($k_{\text{cat}}/K_M < 1$) of coumarin derivative 1g was unexpected. An explanation for this is shown by a binding experiment result that precursor 1g has very low binding affinity (K_D of about 129 μM) against albumin. On the other hand, the significantly high activity (at least 1500-folds higher k_{cat}/K_M compared to 1g) of hydrophobic prodrug 1h is likely attributed to the long alkyl chain portion of 1h that facilitates the entry into the hydrophobic binding pocket.

[0117]

Next, a series of experiments were performed in order to evaluate the *in cellulo* activity of the anticancer approach based on GAR_M.

Cytotoxicity test was performed by using prodrug 1h at set particular concentrations (32 μM for SW620 human colon adenocarcinoma cells; 64 μM for HeLa human cervical cancer-derived cells and A549 human pulmonary alveolar basement epithelial adenocarcinoma cells) and changing the amount of GAR_M-Ru1 added.

[0118]

Cell survival rate measurement was performed with CellTiter 96TM Aqueous One Solution Cell Proliferation Assay (MTS) from Promega. Cultured cells were seeded in a FalconTM 96-well microplate at a density of about 10^3 cells/well, and cultured overnight. Subsequently, the medium was removed, the compounds at various concentrations were added. DMSO was used for dissolving the compounds, and the DMSO concentration was 1% for addition to the cells. After incubating for 96 hours, the cell culture medium was substituted with 85 μL of fresh medium. Subsequently, 15 μL of MTS reagent was added, this was incubated at 37°C for 2.5 hours, and then absorbance was measured at 490 nm. The absorbance of cells with addition of 1% DMSO as the control was set at 100%, and the cell survival rate was calculated.

In order to explain the targeting effect by sugar chains, experiments under similar conditions were performed with alb-Ru1

without sugar chain as the control. From these results, in all three cancer cell strains, the mixture of prodrug 1h and GArM-Ru1 significantly decreased cell proliferation ($< 5\%$), and the effect surpassed the effect at corresponding concentrations of prodrug 1h and GArM-Ru1 (Figure 11). Another important observation was that the cytotoxic activity of the mixture of prodrug 1h and alb-Ru1 was far lower in effectiveness. This suggests that targeting by sugar chains is essential for localization of metal catalyst to the cell surface or inside cells.

[0119]

[Example 7]

Synthesis of artificial metalloenzyme (ArM) that detects ethylene (ArM ethylene probe; AEP)

[0120]

Figure 12 describes the synthetic scheme of ArM ethylene probe (AEP).

Fluorescence emitted by 7-diethylamino coumarin (DEAC) per se has high sensitivity to the polarity of the surrounding solvent, and the quantum yield of DEAC-Ru increases to about 20-folds by the change from being under a polar environment (10% dioxane/water) to a nonpolar environment (60% dioxane/water). Accordingly, in this synthesis, AEP was synthesized based on the reaction between DABCYL inactivating agent comprising olefin (quencher) and alb-Ru.

[0121]

7-1. Preparation of AEP

Thirty micromolar HSA solution (167 μL from 50 nmol, 300 μM aqueous solution) and 37 μM DEAC-Ru solution (167 μL from 62 nmol, 370 μM dioxane solution) were mixed to prepare alb-Ru. The reaction solution was filled to a total amount of 1670 μL with pH 7.4 PBS buffer solution comprising 10% dioxane. After starting the reaction by addition of HSA, the reaction solution was gently stirred and incubated at 37 degrees for 1 hour. Subsequently, the reaction solution was concentrated with Amicon Ultra Centrifugal

filter (30 kDa), and washed three times with PBS buffer solution. Water was added to the concentrated alb-Ru solution to dilute to 50 μ L, and 1 mM stock solution was obtained. In order to prepare AEP solution, a mixed solution of 100 μ M alb-Ru solution (50 μ L from 50 nmol, 1 μ M aqueous solution) and 500 μ M DABCYL quencher solution (450 μ L from 250 nmol, 555 μ M DMSO:water = 1:8 solution) was made. The reaction solution was gently stirred and incubated at 37 degrees for 5 minutes. Subsequently, the reaction solution was concentrated with Amicon Ultra Centrifugal filter (30 kDa), and washed three times with PBS buffer solution. Water was added to the concentrated AEP solution to dilute to 500 μ L, and 100 μ M stock solution was obtained.

[0122]

The fluorescence intensity of the protein complex (alb-Ru, AEP) was measured with JASCO FP-6500 Spectrofluorometer equipped with JASCO FMP-963 microplate reader. In order to prepare samples, both alb-Ru and AEP were prepared into aqueous solutions at a concentration of 10 μ M. The samples were then aliquoted (100 μ L) into a 96-well microplate and measured at $\lambda_{EX} = 420$ nm/ $\lambda_{EM} = 463$ nm. All measurements were performed three times.

[0123]

Moreover, circular dichroism (CD) analysis was performed in order to distinguish the major change in the structure of the protein complex (alb-Ru, AEP) employed in this research. For CD spectrum, 0.1 cm cells were used, and J-1500 Circular Dichroism Spectrometer (JASCO) was employed for measurement. A 10% dioxane aqueous solution was used as the blank, and this was automatically subtracted from the sample during scan. Data was recorded for 200 - 250 nm at a scan speed of 100 nm/min. The concentration of each protein complex was retained at 2.3 μ M.

[0124]

[Result]

The RuQ complex (DEAC-Ru-DABCYL complex) present in the binding pocket of albumin had a significantly low fluorescence intensity due to the quencher, and this is also clear from comparing the fluorescence intensity of alb-Ru and AEP probe in

Figure 13. Further, since the circular dichroism (CD) of these completely overlapped, it was confirmed that no major structure degeneration in the protein was seen (Figure 14).

[0125]

7-2. Detection of ethylene by AEP

The ethylene detection mechanism by AEP is by reacting ethylene with the ruthenium catalyst in AEP to allow substitution with the DABCYL quencher and activation of the fluorescence signal. Accordingly, in this Example, AEP was employed for detection of ethylene in fruits and plants.

[0126]

[Imaging of kiwi fruit]

Immature or mature kiwi fruits were purchased at a grocery store. In order to obtain fragments comprising the outer pericarp, loculus, and columella, kiwi fruits were cut into about 2.0 cm x 4.5 cm sizes with a kitchen knife to prepare kiwi fruit fragments. In order to track ethylene production, 170 μL of AEP (400 μM solution) was poured in the center of a 10-cm dish. Subsequently, the samples were allowed to act on the AEP solution. The samples were incubated at room temperature, and imaging was performed after a certain amount of time (after 1, 24 hours). Keyence BZ-X710 All-in-one Fluorescence Microscope™ equipped with ET-EBFP2/Coumarin/Attenuated DAPI Filter Set Cat# 49021 (Chroma Technology Corp.) was employed for imaging. Bright field image (color) and the fluorescent image were obtained at exposure settings of 1/25 seconds and 1/3.5 seconds, respectively. Multiple imaging images were obtained at 4 X magnification, image binding and analysis was performed with BZ-X Analyzer software (Keyence).

[0127]

[Imaging of *Arabidopsis thaliana*]

Arabidopsis thaliana was grown in soil at 23 degrees with light intensity of 85 $\mu\text{mol m}^{-2}\text{s}^{-1}$. Photoperiodicity of 10 hours of light and 14 hours of dark was applied. In order to track ethylene production, leaves were first taken from 4 to 6-week

plants. Subsequently, the transparent epidermal skin was peeled from the leaf with a clamp, and placed in a 96-well plate with a transparent bottom. After adding water (100 μ L), samples were incubated at room temperature for 12 hours in order to eliminate the effect of damage stress. Depending on the experiment condition, various solutions such as 1 mM ACC solution (2 μ L of 55 mM aqueous solution), 4.8 μ M flg22, or elf18 solution (5 μ M, 100 μ M aqueous solution), and OD₆₀₀ = 0.02 *Pseudomonas* bacteria solution (2 μ L of OD₆₀₀ = 1.0 standard solution) were added as necessary. In an experiment comprising ACC and *Pseudomonas* bacteria, 12 hours of incubation at room temperature was applied. On the other hand, in an experiment comprising PAMP, 6 hours of incubation at room temperature was applied. Subsequently, these solutions were completely removed from epidermal skin samples, and 100 μ M AEP solution (50 μ L from stock solution) was added. After 30 minutes, the solution was completely removed and washed with water, and then imaging was performed with Keyence BZ-X710 All-in-one Fluorescence Microscope™ equipped with ET-EBFP2/Coumarin/Attenuated DAPI Filter Set Cat# 49021 (Chroma Technology Corp.). Imaging images were obtained at 20 X, 40 X magnification, and the bright field image (monochrome) and the fluorescent image was obtained at exposure settings of 1/400 seconds and 1/30 seconds, respectively. Multiple imaging images were obtained at 4 X magnification, and analysis was performed with BZ-X Analyzer software (Keyence).

[0128]

[Result]

Spatial detection of ethylene in fruits

In general, in climacteric fruits, production of autocatalytic ethylene (system 2) progresses during the process of maturing. This is similar for external stimulation such as damage stress or pathogen infection. In this research, endogenous ethylene was first examined by an AEP probe. In kiwi fruits, it is reported that during maturing, the amount of ethylene produced is increased in the outer pericarp via upregulation of the ACS isogene. In an imaging experiment of kiwi fruit with AEP, focus

was placed on observing the change in the amount of ethylene production expressed in various organelles (the outer pericarp, loculus, and columella) in different developmental stages (immature and mature). As summarized in Figure 15, as the fruit matures from the immature state, significant increase in fluorescence intensity was observed in the outer pericarp. On the other hand, the change in fluorescence intensity in the loculus and columella of kiwi fruit was not observed so much. From these results, it became clear that the use of AEP is a promising means for detecting ethylene expression during mature and maturing period of fruits.

[0129]

Ethylene detection in plants

In order to investigate the effect of AEP detection in plants, a small flowering plant *Arabidopsis thaliana* (Brassicaceae family) was selected as the model plant. In order to show with certainty that it is possible to detect the production of ethylene by APE, a comparison experiment was performed with a low-molecular known to control ethylene production and various plant variants involved in ethylene production.

[0130]

In this research, *Arabidopsis thaliana* Col-0 ecotype was used as the wild-type model plant. Further, a wide range of various plant variants such as *acs1/2/6/4/5/9/7/11* or *etol-1* were also used. Variant *acs1/2/6/4/5/9/7/11* is a variant with 8 ACS genes knocked out, and the ethylene amount does not increase even when a pathogen invades. This is because ACS is responsible for an important role related to the biosynthetic pathway of ethylene. Further, *etol-1* which excessively produces ethylene was employed as another variant. Proteasome-dependent degradation is suppressed through the expression of ethylene-overexpression protein 1 (ET01), and ACS activity is positively controlled. This is based on interaction between the C-terminal of type II ACS and ET01.

[0131]

Imaging images of Col-0 incubated at room temperature with or without addition of AEP are shown in Figure 16. As quantified in Figure 17, significant increase in fluorescence intensity was observed, thereby confirming that AEP can be utilized for ethylene detection in the epidermal skin of *Arabidopsis thaliana*. As positive control, comparison was made with (an ethylene product of) Col-0 externally stimulated by ACC. As anticipated, a higher increase in fluorescence intensity was shown compared to wild-type Col-0.

[0132]

[Example 8]

Selective tagging of cRGD against cancer cells by glycosylated ArM

It has been reported in many researches that cyclic Arg-Gly-Asp (cRGD) pentapeptide inhibits $\alpha v \beta_3$ and $\alpha v \beta_5$ integrins overexpressed on the cancer cell surface, and is effective for preventing adherence to the extracellular matrix (J. S. Desgrosellier, D. A. Cheresh, Integrins in cancer: Biological implications and therapeutic opportunities. Nat. Rev. Cancer 10, 9-22 (2010).; M. Pfaff, K. Tangemann, B. Muller, M. Gurrath, G. Muller, H. Kessler, R. Timpl, J. Engel, Selective recognition of cyclic RGD peptides of NMR defined conformation by $\alpha IIb \beta_3$, $\alpha v \beta_3$, and $\alpha 5 \beta_1$ integrins. J. Biol. Chem. 269, 20233-20238 (1994).; and M. Aumailley, M. Gurrath, G. Muller, J. Calvete, R. Timpl, H. Kessler, Arg-Gly-655 Asp constrained within cyclic pentapeptides. Strong and selective inhibitors of 656 cell adhesion to vitronectin and laminin fragment P1. FEBS Lett. 291, 50-54 657 (1991).). Moreover, it has been shown that an RGD-based antagonist against integrins expressed on vascular endothelial cells facilitates tumor by inhibiting neovascularization (P. C. Brooks, A. M. P. Montgomery, M. Rosenfeld, R. A. Reisfeld, T. Hu, G. Klier, D. A. Cheresh, Integrin $\alpha v \beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 79, 1157-1164 (1994).; and D. G. Stupack, D. A. Cheresh, Integrins and angiogenesis. Curr. Top. Dev. Biol. 64, 207-238 (2004).).

In this Example, it was investigated whether glycosylated ArM (GArM) may be used for selective cell tagging (SeCT) therapy for destroying adherence of cancer cells at single cell level, which mimics the seeding process of micrometastasis. Specifically, it was verified whether this is possible by selectively accumulating GArM complex targeting HeLa to cancer cells, and then selectively tagging cRGD to surface proteins.

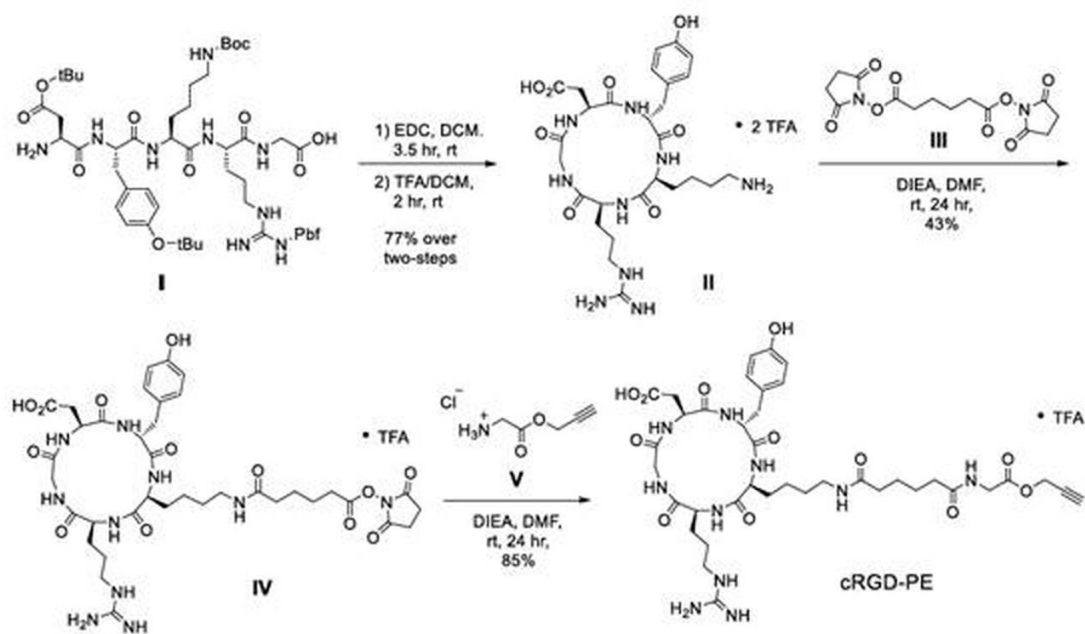
[0133]

8-1. Preparation of cRGD-PE

The scheme employed for preparation of cRGD-PE was as follows:

[0134]

[Chemical Formula 9]



[0135]

(Preparation of compound I)

Synthesis was performed by a standard solid phase peptide synthesis method. After cutting out from the solid phase support, the mixture was directly purified by a reverse phase HPLC with a linear gradient condition of 20 - 80% acetonitrile aqueous solution (0.1% TFA) over 40 minutes. Yield: 2.92 g, 89%, ESI-HRMS m/z calcd for $C_{53}H_{83}N_9O_{14}S$ ($[M+H]^+$) 1102.5853, found 1102.5859.

[0136]

(Preparation of compound II)

Compound I (90.1 mg, 0.0833 mmol) and EDC (17.6 mg, 0.0916 mmol) were dissolved in methylene chloride (0.5 mL). Subsequently, the solution was stirred at room temperature for 3.5 hours. For workup, methylene chloride was added, the organic layer was washed with water/saturated saline, dried with magnesium sulfide, and concentrated under reduced pressure. Production of the target substance was confirmed by mass spectrometry of the residue obtained. ESI-HRMS m/z calcd for $C_{53}H_{81}N_9O_{13}S$ ($[M+H]^+$) 1084.5747, found 1084.5744.

Subsequently, this protected peptide obtained was dissolved in a solution of TFA/methylene chloride (1:1) for 2 hours to perform deprotection. The mixture was concentrated under reduced pressure, and directly purified by a reverse phase HPLC with a linear gradient condition of 20 - 80% acetonitrile aqueous solution (0.1% TFA) over 40 minutes. Yield: 54.5 mg, 77% (2-step yield). ESI-HRMS m/z calcd for $C_{27}H_{41}N_9O_8$ ($[M+H]^+$) 620.3151, found 620.3169.

[0137]

(Preparation of compound III)

A DMF solution (20 mL) of adipic acid (1.0 g, 6.84 mmol) was stirred, and N-hydroxysuccinimide (3.0 g, 27.4 mmol) and EDC (5.14 g, 27.4 mmol) were added. After stirring the solution at room temperature for 24 hours, the reaction mixture was concentrated under reduced pressure, the crude product was dissolved in 200 mL of acetone, and 250 mL of 1 M hydrochloric acid aqueous solution was added dropwise. After 2 hours, white precipitate was filtered, and washed with water and acetone to obtain the target substance (1.75 g, 77%). 1H NMR (500 MHz, $CDCl_3$, 25°C) δ 2.84 (s, 4H), 2.83 (s, 4H), 2.67 (t, J = 3.5 Hz, 4H), 1.89 (t, J = 3.5 Hz, 4H); ^{13}C NMR (125 MHz, $CDCl_3$, 25°C) 169.4, 168.4, 30.7, 25.9, 23.9. ESI-HRMS m/z calcd for $C_{14}H_{17}N_2O_8$ ($[M+H]^+$) 341.0979, found 341.0973.

[0138]

(Preparation of compound IV)

A DMF solution (680 mL) of compound II (28.8 mg, 0.034 mmol) and compound III (23.2 mg, 0.068 mmol) was stirred, and DIEA

(16.8 μ L, 0.10 mmol) was added. After stirring the solution at room temperature for 24 hours, and the mixture was directly purified with a reverse phase HPLC with the same condition as that employed in the preparation of compound I to obtain the target compound (14.0 mg, 43%). ^1H NMR (500 MHz, CD_3OD , 25°C) δ 7.00 (d, J = 6.5 Hz, 2H), 6.68 (d, J = 6.5 Hz, 2H), 4.74 (t, J = 7.0 Hz, 1H), 4.41 (q, J = 7.0 Hz, 1H), 4.26-4.30 (m, 2H), 3.91-3.86 (m, 1H), 3.22-3.18 (m, 1H), 3.14-3.10 (m, 1H), 3.09 (t, J = 6.5 Hz, 2H), 2.91-2.85 (m, 2H), 2.84-2.78 (m, 5H), 2.59 (t, J = 9.5 Hz, 2H), 2.64-2.60 (m, 1H), 2.58 (dd, J = 16.5, 9.0 Hz, 1H), 2.19 (t, J = 7.0 Hz, 2H), 2.22 (t, J = 7.0 Hz, 2H), 1.92-1.82 (m, 1H), 1.74-1.60 (m, 6H), 1.57-1.44 (m, 3H), 1.44-1.32 (m, 2H), 1.09-0.90 (m, 2H); ESI-HRMS m/z calcd for $\text{C}_{37}\text{H}_{53}\text{N}_{10}\text{O}_{13}$ ($[\text{M}+\text{H}]^+$) 845.3788, found 845.3769.

[0139]

(Preparation of cRGD-PE)

A DMF solution (200 μ L) of compound IV (4.2 mg, 3.9 μ mol) and compound V (1.1 mg, 7.8 μ mol) was stirred, and DIEA (3.3 μ L, 19.6 μ mol) was added. After stirring the solution at room temperature for 24 hours, the mixture was directly purified with a reverse phase HPLC with the same condition as that employed in the preparation of compound I to obtain cRGD-PE (3.2 mg, 85%). $\delta^1\text{H}$ NMR (500 MHz, CD_3OD , 25°C) 7.02 (d, J = 7.0 Hz, 2H), 6.71 (d, J = 7.0 Hz, 2H), 4.76 (t, J = 7.5 Hz, 1H), 4.75 (s, 2H), 4.44 (t, J = 7.5 Hz, 1H), 4.29-4.20 (m, 2H), 3.98 (s, 2H), 3.91 (dd, J = 9.5, 3.0 Hz, 1H), 3.25-3.20 (m, 1H), 3.18-3.11 (m, 1H), 3.11 (t, J = 7.0 Hz, 2H), 2.96 (s, 1H), 2.88 (d, J = 8.0 Hz, 2H), 2.85 (dd, J = 16.5, 8.0 Hz, 1H), 2.58 (dd, J = 16.5, 8.0 Hz, 1H), 2.29 (t, J = 7.0 Hz, 2H), 2.22 (t, J = 7.0 Hz, 2H), 1.92-1.84 (m, 1H), 1.74-1.60 (m, 6H), 1.59-1.42 (m, 3H), 1.41 (t, J = 8.0 Hz, 2H), 1.09-0.80 (m, 2H); ESI-HRMS m/z calcd for $\text{C}_{38}\text{H}_{55}\text{N}_{10}\text{O}_{12}$ ($[\text{M}+\text{H}]^+$) 843.3995, found 843.4006.

[0140]

8-2. Preparation of GArM complex

Using a previously reported method, a sugar chain-aldehyde probe having a terminal $\alpha(2,3)$ -sialic acid (R. Sibgatullina, K. Fujiki, T. Murase, T. Yamamoto, T. Shimoda, A. Kurbangalieva, K. Tanaka, Highly reactive "RIKEN click" probe for glycoconjugation on lysines. *Tetrahedron Lett.* 58, 1929-1933 (2017).), as well as a gold catalyst bound to coumarin (K. Tsubokura, K. K. Vong, A. R. Pradipta, A. Ogura, S. Urano, T. Tahara, S. Nozaki, H. Onoe, Y. Nakao, R. Sibgatullina, A. Kurbangalieva, Y. Watanabe, K. Tanaka, In vivo gold complex catalysis within live mice. *Angew. Chem. Int. Ed.* 56, 3579-3584 (2017).) were synthesized.

[0141]

To a human serum albumin solution (5.3 mL of aqueous solution, 66.7 nmol), a solution of a sugar chain-aldehyde probe having a terminal $\alpha(2,3)$ -sialic acid in DMSO (1 μ mol, 15 equivalents, 260 μ L from 3.8 mM stock solution) was added under atmosphere. The solution was gently stirred, and incubated overnight at 37°C. Subsequently, the solution was concentrated with Amicon Ultra Centrifugal filter (30 kDa), and washed with water. After removing the insoluble byproduct with Durapore membrane (Durapore PVDF 0.45 μ mTM), water was added for dilution to obtain 1.0 mM stock solution of sugar chain albumin. Confirmation of the number of bound sugar chains was performed with MALDI-TOF-MS (positive mode), and the molecular weight of sugar chain albumin having 6.2 molecules of sugar chains bound per 1 molecule of albumin was thereby detected (85.8 kDa). In the next step of the protocol, to a solution of sugar chain albumin solution (66.7 nmol) in PBS buffer (60.6 μ L, pH 7.4), a solution of coumarin-gold complex (66.7 nmol) in DMSO (6.1 μ L) was added. The solution was gently stirred and incubated at 37 degrees for 1 hour, after which the solution was concentrated with Amicon Ultra Centrifugal filter (30 kDa), and washed with PBS buffer solution to obtain the target GArM complex.

[0142]

(Cells)

Because HeLa cancer cells are known to overexpress RGD-specific integrins ($\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$), HeLa cells were selected as

the model in this research. HeLa cells employed were provided from the Cell Engineering Division -CELL BANK- at RIKEN.

[0143]

HeLa cells were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. HeLa-Luc cell strain was made by stable transfection of HeLa cells by firefly luciferase and puromycin acetyl transferase cultured in DMEM comprising 10% FBS and 0.01% puromycin. HeLa-V cell strain was made by stable transfection of HeLa cells by firefly luciferase and Venus (V) cultured in DMEM comprising 10% FBS, 1% penicillin-streptomycin, and 0.8% Geneticin. All cell strains were allowed to propagate in an incubator at 37°C containing 5% carbon dioxide. As previously described (A. Ray, B. N. Dittel, Isolation of mouse peritoneal cavity cells. JoVE, e1488 723 (2010).), mouse macrophage was collected from the peritoneal cavity of BALB/c-nu/nu mice administered with 0.9% saline (6 mL).

[0144]

8-3. Verification of selectivity of GArM towards HeLa cell

In order to confirm whether GArM can more selectively target HeLa cells, a series of flow cytometry research was performed. Since the fluorescence intensity of coumarin derivative is known to increase when bound to albumin, fluorescence intensity measured in flow cytometry at $\lambda_{\text{Ex}} = 405 \text{ nm}$ / $\lambda_{\text{Em}} = 470 \text{ nm}$ will be an indicator of whether the coumarin-bound GArM complex bound to the cells.

[0145]

Flow cytometry and cell sorting was performed with Sony SH800 Cell Sorter (Sony Corporation) by a common method. The flow cytometer was equipped with 405, 488, 561, and 638 nm lasers, excitation/emission wavelength gates of $\lambda_{\text{Ex}} = 405 \text{ nm}$ / $\lambda_{\text{Em}} = 470 \text{ nm}$ for GArM detection and $\lambda_{\text{Ex}} = 515 \text{ nm}$ / $\lambda_{\text{Em}} = 528 \text{ nm}$ for Venus (V) detection were each employed, and the result was analyzed with Sony SH800 software.

[0146]

First, in the cell culture system, an apparent difference in peak was seen in flow cytometry between GArM-treated HeLa cells and untreated HeLa cells (Figure 18). By mixing GArM-treated HeLa cells and untreated HeLa cells, this characteristic was emphasized more, and peaks that are clearly defined as two peaks were seen.

[0147]

Next, as a control experiment, the binding ability of GArM was examined with macrophages extracted from the peritoneal cavity of mice. Comparing the flow cytometry histogram (Figure 19), only a slight difference was observed between macrophages with and without addition of GArM, and this, as anticipated, suggests that there is no or almost no binding.

[0148]

In a final test that verifies selectivity, the combinations of macrophage and HeLa cells incubated with or without addition of GArM were observed (Figure 20). In this experiment, in order to observe HeLa cells and macrophage with discrimination, HeLa-V cell strain expressing Venus (V) yellow fluorescent protein was used. From the flow cytometry profile, Venus-expressing HeLa cells and macrophages can be clearly discriminated. It was found that when GArM is added, the majority of the HeLa-V cells migrate to the top right portion which shows GArM binding. On the other hand, such change was not seen with macrophages. As a result, this data strongly indicates that GArM can preferentially bind to HeLa cancer cells at the cell level.

[0149]

8-4. Cell adhesion assay

The ability of SeCT labeling reagent (GArM/cRGD-PE) that impedes cell adhesion based on integrin was confirmed by *in vitro* cell adhesion assay. Cell adhesion assay was performed with Human Fibronectin Coated 96-Well Microplates purchased from R&D System (Minneapolis, U.S.A.).

[0150]

The adhered cells were quantified with a commercially available MTS assay CellTiter 96 Aqueous One Solution cell

proliferation assay (Promega). HeLa cells employed in this experiment were made serum free 16 hours before the experiment by exchanging the proliferation medium to DMEM (serum-free). Subsequently, cells were subcultured to a concentration of 6×10^5 cells/mL stock solution, and the remaining trypsin was removed by further centrifugation. Next, a mixed solution was prepared in an Eppendorf tube with HeLa cells (360 μ L from cell stock solution), cRGD-PE (45 μ L from 0, 160, 320, 640, 1280, 2560, 5120, 10240 μ M stock aqueous solution), and GAR_M (45 μ L from 400 μ M PBS buffer solution). In parallel, GAR_M is substituted to 45 μ L of PBS buffer solution only to prepare a control solution. Incubation was performed at room temperature for 1 hour with a rocking shaker. Next, a washing step of spinning down the cells (at 0.8 ppm for 4 minutes) and removing the supernatant fluid, and then resuspending in 450 μ L of DMEM only (serum-free) was performed two times. In a fibronectin-coated 96-well microplate, 130 μ L of labeled HeLa cell mixture was added to each well, and subsequently, this plate was incubated at 37°C for 10 minutes. After removing the medium, the adhered cells were washed 3 - 4 times with PBS buffer solution. PBS buffer solution was intermittently pipetted up and down in order to remove non-specifically bound cells. In the final step, 100 μ L of DMEM (10% FBS + 1% penicillin-streptomycin) and 20 μ L of MTS reagent were added. After incubating at 37°C for 4 hours, the endpoint absorbance at 490 nm was obtained with SpectraMax iD3 multi-mode microplate reader (Molecular Devices, U.S.A.).

[0151]

The result is shown in Figure 21. As anticipated, a significant difference in cell adhesion on fibronectin-coated plates was observed for HeLa cells treated with SeCT labeling reagent (GAR_M/cRGD-PE). These differences were in particular more apparent when the cRGD-PE treatment concentration is high, and this indicates that cRGD catalytically bound on the cell surface has a higher concentration.

CLAIMS

1. A complex of a protein and a catalyst selected from metal catalysts or organic catalysts, wherein
said protein is a protein that has a hydrophobic pocket within its three-dimensional structure, and
said complex accommodates said catalyst in said hydrophobic pocket so that said catalyst is not exposed or not substantially exposed to the hydrophilic environment.
2. The complex according to claim 1, wherein said protein is a natural or artificial protein.
3. The complex according to claim 1 or 2, wherein said protein is selected from the group consisting of human serum albumin (HSA), immunoglobulin G (IgG), immunoglobulin A (IgA), transferrin, antitrypsin, haptoglobin, α 1-acidic glycoprotein, Myoferlin, Trk receptor, estrogen receptor, and folate receptor.
4. The complex according to any one of claim 1 to 3, wherein said metal catalyst is selected from the group consisting of a boron catalyst, a magnesium catalyst, an aluminum catalyst, a silicon catalyst, a calcium catalyst, a scandium catalyst, a titanium catalyst, a vanadium catalyst, a chromium catalyst, a manganese catalyst, an iron catalyst, a cobalt catalyst, a nickel catalyst, a copper catalyst, a zinc catalyst, an yttrium catalyst, a zirconium catalyst, a niobium catalyst, a molybdenum catalyst, a ruthenium catalyst, a rhodium catalyst, a palladium catalyst, a silver catalyst, an indium catalyst, a tin catalyst, a barium catalyst, a hafnium catalyst, a tungsten catalyst, a rhenium catalyst, an osmium catalyst, an iridium catalyst, a platinum catalyst, a gold catalyst, and a lanthanoid Lewis acid catalyst.
5. The complex according to claim 4, wherein said lanthanoid Lewis acid catalyst is selected from the group consisting of an ytterbium catalyst, a lanthanum catalyst, a cerium catalyst, a

samarium catalyst, an europium catalyst, a gadolinium catalyst, a terbium catalyst, a thulium catalyst, and a lutetium catalyst.

6. The complex according to any one of claim 1 to 5, wherein said protein is a human serum albumin, and said catalyst is a metal catalyst.

7. The complex according to claim 6, wherein said metal catalyst is a ruthenium catalyst.

8. The complex according to claim 6 or 7, wherein said hydrophobic pocket of human serum albumin is the albumin drug binding site I (drug site I).

9. The complex according to any one of claim 6 to 8, wherein said metal catalyst is bound to the said hydrophobic pocket via a ligand against human serum albumin.

10. The complex according to claim 9, wherein said ligand is selected from the group consisting of warfarin, azapropazone, acenocoumarol, phenylbutazone, salicylate salt, indomethacin, phenytoin, tolbutamide, chlorpropamide, iophenoxate, iodipamide, sulfadimethoxine, phenprocoumon, glibenclamide, sulfathiazole, tenoxicam, camptothecin, balzidendeazi, andelleratan, diadelleal, diadellealtan, prodan, bilirubin, eicosanoid, and carboxy-methyl-propyl-furanpropanoate (uremic toxin), and coumarin.

11. The complex according to claim 9 or 10, wherein said metal catalyst is bound to the said hydrophobic pocket via a linker bound to said ligand.

12. The complex according to claim 11, wherein said linker is an alkyl chain or a polyethylene glycol (PEG) chain having amino and carboxyl groups on both ends.

13. The complex according to claim 12, wherein said linker is C₁ - C₃ alkyl.
14. The complex according to any one of claim 1 to 13, wherein the surface of said protein is modified so as to interact with a target site in vivo.
15. The complex according to claim 14, wherein said modification is a modification by a sugar chain.
16. The complex according to any one of claim 1 to 13, wherein said protein further comprises a portion that interacts with a target site in vivo.
17. The complex according to claim 14 or 15, wherein said protein is human serum albumin.
18. A composition comprising the complex according to any one of claim 1 to 17.
19. The composition according to claim 18, which is a pharmaceutical composition further comprising a pharmaceutically acceptable carrier.
20. The composition according to claim 19, which is used in combination with a prodrug that can be activated by said complex.
21. The composition according to claim 19, further comprising a prodrug that can be activated by said complex.
22. The composition according to claim 19, which is employed for selectively tagging particular cells.
23. The composition according to claim 22, which is administered in combination with a chemical substance that is tagged to said cells.

24. The composition according to claim 18, which is used as a biosensor.

25. The composition according to claim 18, which is used as a biosensor for detecting ethylene.

26. A pharmaceutical composition comprising a prodrug, wherein
said prodrug can be activated by the complex according to
any one of claim 1 to 17, and
said pharmaceutical composition is used in combination with
the complex according to any one of claim 1 to 17.

27. A combination medicine comprising
a first agent comprising the complex according to any one
of claim 1 to 17, and
a second agent comprising a prodrug that can be activated
by said complex.