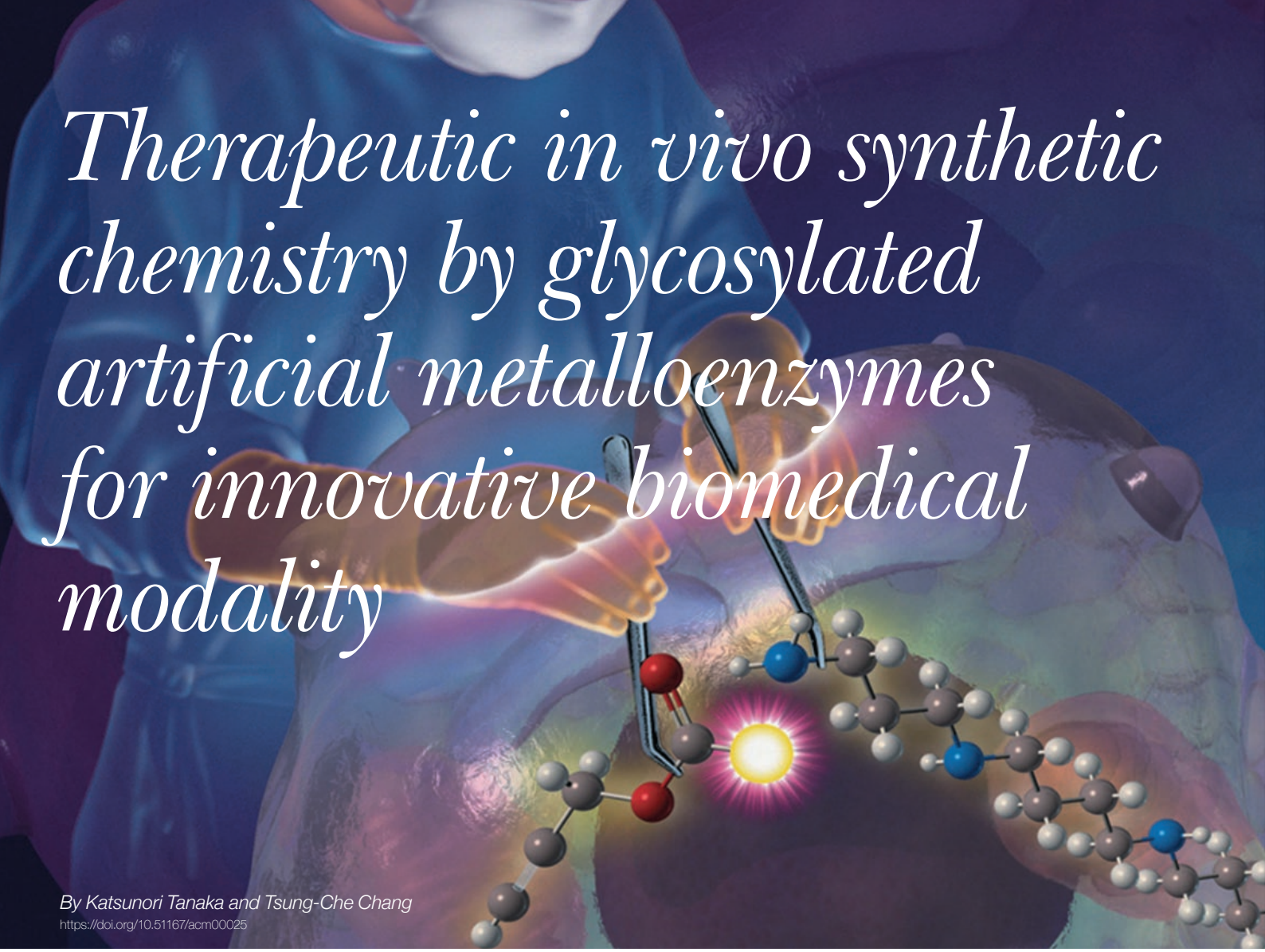
Therapeutic in vivo synthetic chemistry by glycosylated artificial metalloenzymes for innovative biomedical modality



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https://doi.org/10.51167/acm00025

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This article is to depict the steps taken by our team for the development of glycosylated artificial metalloenzymes (GArMs) that we have used to develop therapeutic in vivo synthetic chemistry. To achieve this goal, we have had to combine technologies developed over the course of a decade that range from protein conjugation methodologies, identification of glycan-dependent targeting, development of functional biocatalysis and the biocompatible reactions. As a result, we have begun to reveal the framework for GArM complexes and their potential towards creating novel biotechnological tools and therapeutic applications.

Therapeutic In Vivo Synthetic Chemistry

In the past century, synthetic chemistry, which is the artificial execution of various chemical reactions to obtain the desired products, has greatly enabled the development of pharmaceutical research, leading to an improvement in the health of patients worldwide. Recently, innovations in new synthetic methods, biocatalysis, reaction miniaturization, and chemoinformatics have powerfully improved the quality of products in pharmaceutical research. One of the most influential research trends within the last few decades was the race to discover anticancer drugs. However, the adverse health effects of current anticancer drugs have sparked a new research trend centered on localized drug delivery to avoid unwanted side effects against untargeted normal cells.[1] Currently, numerous research groups are developing vastly different approaches to overcome this issue. Some notable approaches (Fig. 1A-D) include the use of external stimuli (ex/MRI, ultrasound, and light)[2], and bioorthogonal click-to-release chemistry[3] to convert prodrugs into their active form. In addition, pH-sensing liposomes and nanoparticles have extensively explored to release encapsulated drugs under the acidic conditions of cancers[4]. Recently, abiotic

metal-catalyzed uncaging reactions to release drugs have also utilized to cancer treatments due to bioorthogonal character and the outstanding catalytic activity of metals[5]. These approaches, however, still have some drawbacks. For example, the use of external stimuli requires expensive machinery, while click-to-release strategies use abiotic small molecules that need to be directly injected at tumors sites. Since only fully developed tumors are acidic, pH triggered drug release is not as effective for early-stage cancers. Encapsulation of metal nanoparticles can reduce toxicity of abiotic metals in vivo and are accumulated in cancer tissues by enhanced permeability and retention (EPR) effect, however, thousands of research papers gave a critical verdict, that is, the EPR effect works in rodents but not in humans[6]. And, the studies revealed that after treatment, nanomaterials were found to accumulate in the spleen, liver, brain, and lungs to cause oxidative stress via the production of reactive oxygen species (ROS), leading to significant toxicity[7].

Our group offers a vastly different approach by in vivo synthetic chemistry to achieve localized drug synthesis/release on cancers. By definition, in vivo synthetic chemistry is a term used by our group to describe the ability to perform non-natural chemical reactions within living biological systems. Because of the complexity of biological environments, however, a multitude of challenges need to be overcome to achieve the feat. Practically speaking, there are three main areas to address. The first area of focus is related to the targeting methodology. Without proper localization of in vivo synthetic chemistry, this system would be incapable of applicability for biomedical research. The second is the need to develop an effective and biocompatible catalyst for the implementation of in vivo synthetic chemistry. Our group felt that the advantages afforded by abiotic transition metal catalysis, such as the potential for in vivo natural product synthesis, made it an attractive strategy. Lastly, development of biocompatible chemical reactions is also indispensable to the system. In vivo synthetic chemistry does not only require mild and aqueous conditions, but also specific chemoselectively without interfering with biological metabolism. Although our group has only begun to challenge the immense feat, we have identified a path forward using a system that integrates different aspects of our past interesting research (Fig 1E). The article is to highlight the steps taken at each stage, and how they all ultimately fit together for therapeutic in vivo synthetic chemistry.

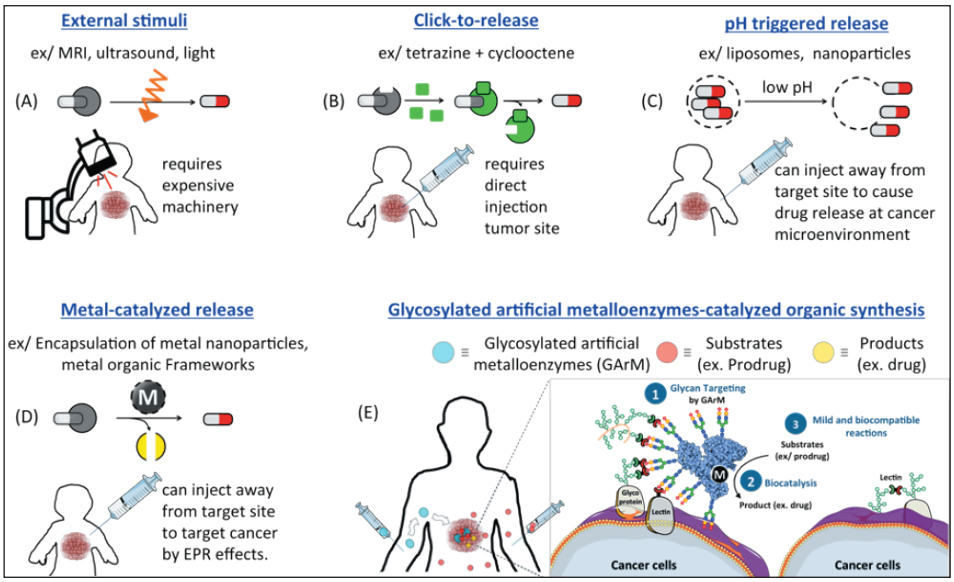

Figure 1. In vivo cancer therapeutic modalities based on strategies of localized drug delivery mediated by (A) external-stimuli responsive systems, (B) click-to-release chemistry, (C) the cancer microenvironment, or (D) the abiotic metal-mediated reactions. (E) Therapeutic in vivo synthetic chemistry via glycosylated artificial metalloenzymes.

RIKEN Click Reaction

As shown in Fig. 2A, the thermal cyclization of 1-azatrienes to 1,2-dihydropyridines via 6π-azaelectrocyclization could be an attractive tool to be utilized for the modification of the lysine amino group on proteins through Schiff-base (imine) formation. However, the requirement for high temperatures and long reaction times for the strategy limited the application of the method in biological systems. Incorporating the interesting chemistry, we found that modification at the C4-carbonyl and C6-alkenyl or phenyl groups in 1-azatrienes enables reducing the energy gap between HOMO and LUMO to significantly accelerate the azaelectrocyclization and occur in a matter of a few minutes at room temperature.[8] Then, We began to shift our interest to developing 6π-azaelectrocyclization for protein labeling. Although many lysine conjugation methodologies were developed at the time, most of them were too slow or not reactive enough. As a result, azaelectrocyclization for lysine-selective conjugation (later coined as the RIKEN click reaction[9-11]) has become a standard technique heavily utilized in our research today. As shown in Figure 2B, we first began to prepare the aldehyde probe directly linked with molecules of interest via amide linkage.[12-18] To simplify the operation of the RIKEN click reaction, we transitioned to using another reaction to link the molecules. This has led to the preparation of RIKEN click reagents modified with groups such as a azide (for Staudinger ligation)[19], dibenzocyclooctyne (for strain-promoted azide-alkyne cycloaddition)[20,21], and *trans*-cyclooctene (for tetrazine ligation)[22-35]. Numerous successfully applied molecules for protein modification clearly prove the versatility of the RIKEN click reaction. For instance, molecular imaging and radiotherapeutic applications have seen the usage of metal chelating agents, such as DOTA[12,13,18,20,30], NOTA[20,30], and closo-decaborate[21]. Moreover, the fluorescent imaging studies have utilized various fluorophores like coumarin[12,14,15], NBD[14,18], TAMRA[12,13,17,23,30], Cy5[13,18,19], Hilyte Fluor 750[16], fluorescein[23], and FRET pairs[24]. Other molecules that have also found significant usage in our research include the conjugation of biotin[17,19,22], and numerous types of complex *N*-glycans[19,22-34].

In terms of conjugates done using the RIKEN click reagent, our studies have shown applicability to various amine-containing scaffolds. A number of peptides and proteins have been conjugated under in vitro conditions; such as the cRGDyK peptide[22,24,30], somatostatin[12,14,22], albumin, orosomucoid[12], and asialoorosomucoid[12]. This has also been extended to dendrimer complexes[18], as well as a number of antibodies that include anti-GFP mAb[12], anti-IGSF4 mAb[20], and trastuzumab[21]. For example, the rapid rate of the RIKEN click reaction has also been beneficial for the preparation of radiotherapeutic agents[20,21]. To approach it, a one-pot reaction can be performed the RIKEN click reagent, a tetrazine-linked metal chelator, and a targeting antibody (Fig. 2C). These radiolabeled antibodies have been shown in mouse models to effectively accumulate to targeted tumors and suppress their growth.

Intriguing, the RIKEN click reagent has also been shown to be applicable in labeling the surface proteins of live cells. For instance, to investigate and identify glycan-dependent mechanisms that could potentially influence in vivo lymphocyte trafficking in living animals, we labeled the lymphocyte that were extracted from nude mice with α(2,6)-sialic acid terminated complex *N*-glycan[19]. The glycosylated lymphocytes were then administered into DLD-1 tumor bearing mice. In the case of glycosylated lymphocytes, observations revealed that besides lymphocyte accumulation in spleen/lymph nodes, detection was also found in implanted tumor regions (Figure 2D). In a control setting, lymphocytes without glycan modifications naturally accumulated to the spleen and intestinal lymph nodes, while no detection was found in the tumor.

Current literature has strongly implicated cancer cell glycosylation to be vital for mediating tumor metastasis and invasion[35]. On the basis of the concept, we first established four kinds of human cancer cells (two cancer cell

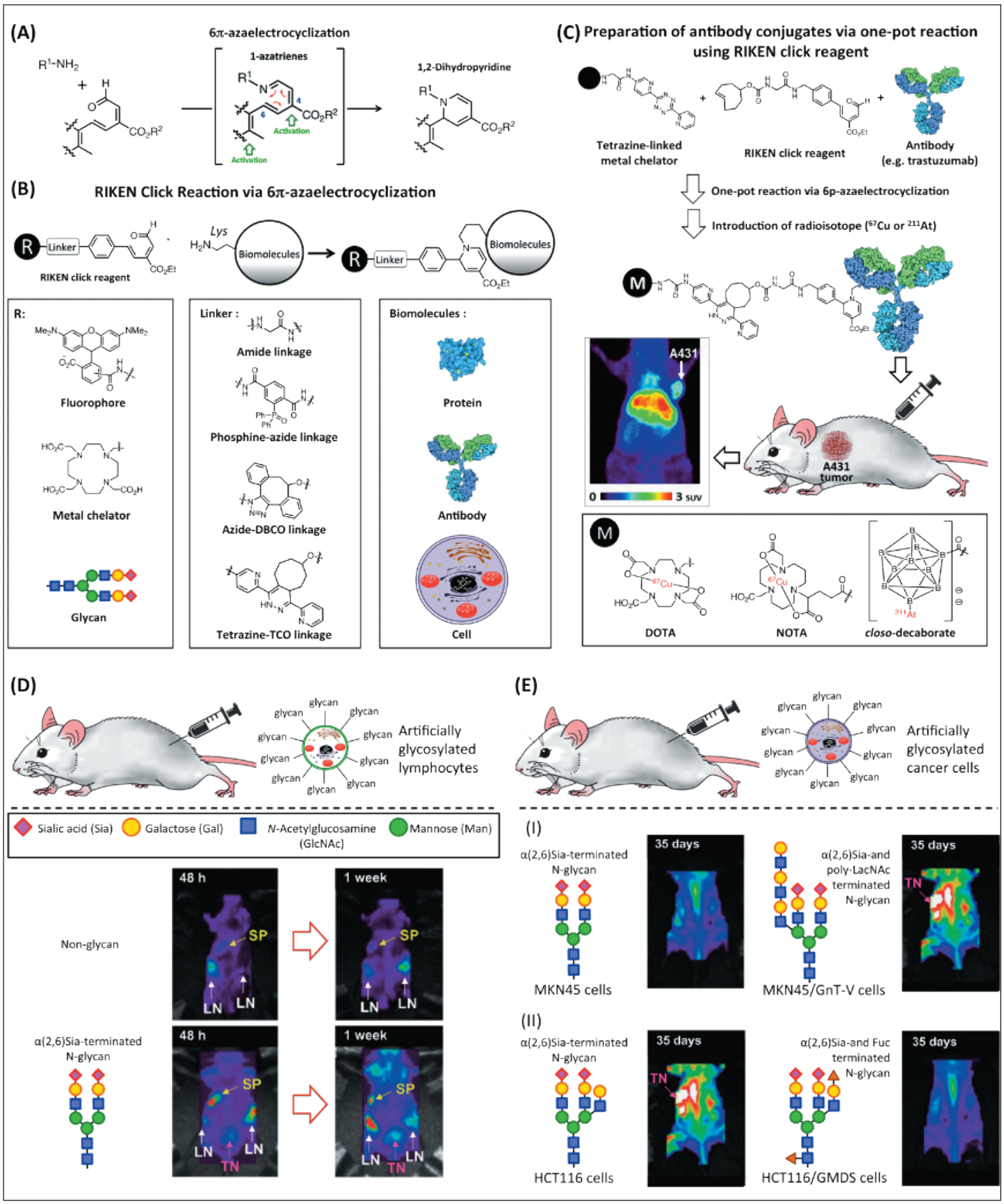

Figure 2. (A) 6p-azaelectrocyclization reaction mechanism from the cyclization of 1-azatrienes to 1,2-dihydropyridine. (B) Applications of the RIKEN click reagent for protein labeling in a number of targeted scaffolds. (C) Preparation of 67Cu-, or 211At-labled radiotherapeutics via a one-pot reaction using the RIKEN click reagent, tetrazine-linked metal chelator, and targeting antibody. In vivo data reveals effective accumulation and suppression of tumor growth. (D) Artificial glycosylated lymphocytes used to study glycan-dependent changes to lymphocyte trafficking. Organs of interest are the spleen (SP), lymph node of the epidermal intestinal tract (LN), and implanted DLD-1 tumor (TN). (E) Artificial glycosylated cancer cells used to study glycan-dependent changes to metastasis. I) Effects of polylactosamine in MKN45-related metastasis. II) Effects of fucosylation in HCT116-related metastasis.

lines, MKN45 and HCT116, and their transfected versions expressing surface glycan-related genes, MKN-GnT-V and HCT116-GMDS). These cancer cells were labeled by RIKEN click reagent linked with Hilyte Fluor 750[16]. Following injection into mice, the in vivo imaging clearly showed that tumor metastasis was dependent upon the cell surface glycans. Namely, polylactosamine structure (Fig. 2E-(I)) or the loss of fucosylation (Fig. 2E-(II)) on the cancer cell surfaces, respectively, enhanced the metastatic potential of the tumor cells. The other tested cells line also include cultures of MDCK[17], HeLa[17,22], HUVEC[23], and RAW264.7 cells[24]. Overall, we have found the RIKEN click reagent to be quite robust and versatile, with its value especially evident when used for labeling large macromolecules.

Glycan Targeting

In nature, one of the major components that drives cell-to-cell interactions is glycan recognition with lecitns. This is due to the fact that many different types of cell surfaces are composed of complex assemblies of glycoproteins, glycolipids, and proteoglycans to regulate their physiological functions (Fig. 3A). Since most types of malignant and diseased cells compared to healthy cells have altered their glycan patterns, this represents a potential targeting mechanism. Individually, lectin-glycan interactions are poor (*Kd* in the mM range), such that their one-to-one interactions have little biological selectivity. However, due to the enormous presence of lectin isoforms, especially in cancer cells, the combined interactions of clustered sugars (i.e., homogeneous vs. heterogeneous) allows for strong and selective cell binding in nature; we refer to the phenomena here as glycan pattern recognition (Fig. 3B). As mentioned above, one issue with conjugating large biomolecules is that conventional protein ligation techniques often suffer from low yields. Therefore, the ability of our RIKEN click reagent to handle the conjugation of complex *N*-glycans makes it a principle approach for the preparation of artificial glycoproteins by decorating various glycan assemblies.

In one of our earlier investigations, homogeneous artificial glycoproteins (**1a-h**) were prepared by using human serum albumin (HSA) as the protein scaffold and injected into mice[27]. As depicted in Figure 3C, a number of observations were made based on the following changes in accumulation and excretion. For instance, glycoproteins **1a-c** were found with prolonged and selective liver accumulation. In contrast, the use

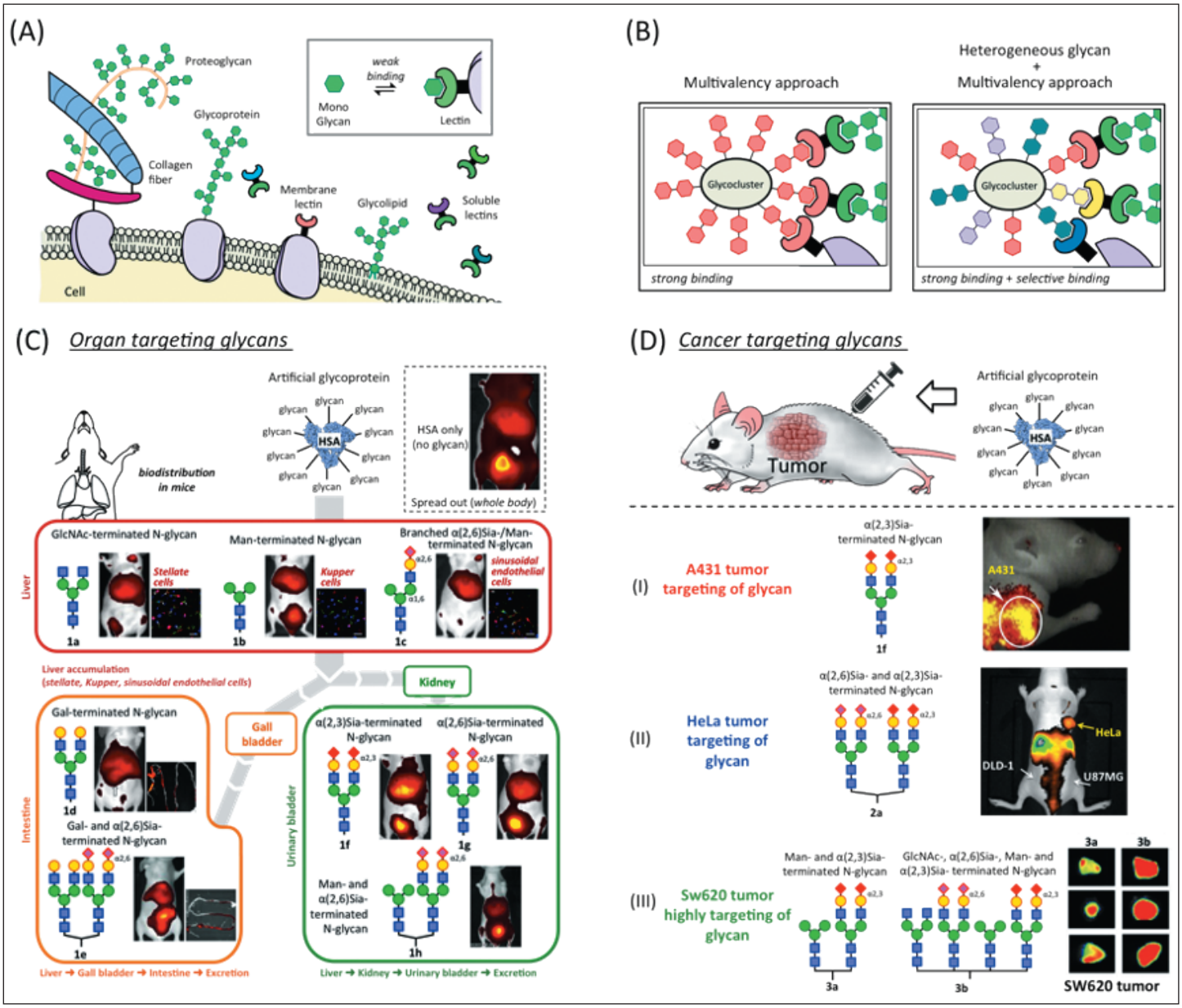

Figure 3. (A) Various type of glycans that exist on cell surfaces. (B) Concept of glycan pattern recognition. In the presence of matching glycan patterns and lectin expression, glycoclusters can exhibit strong and selective cell binding. (C) Imaging studies showing the biodistribution of artificial glycoproteins 1a-h following injection into mice. (D) Imaging studies showing the tumor targeting properties of glycoproteins 1f, 2a, and 3a-b for accumulation to different tumors implanted into mice.

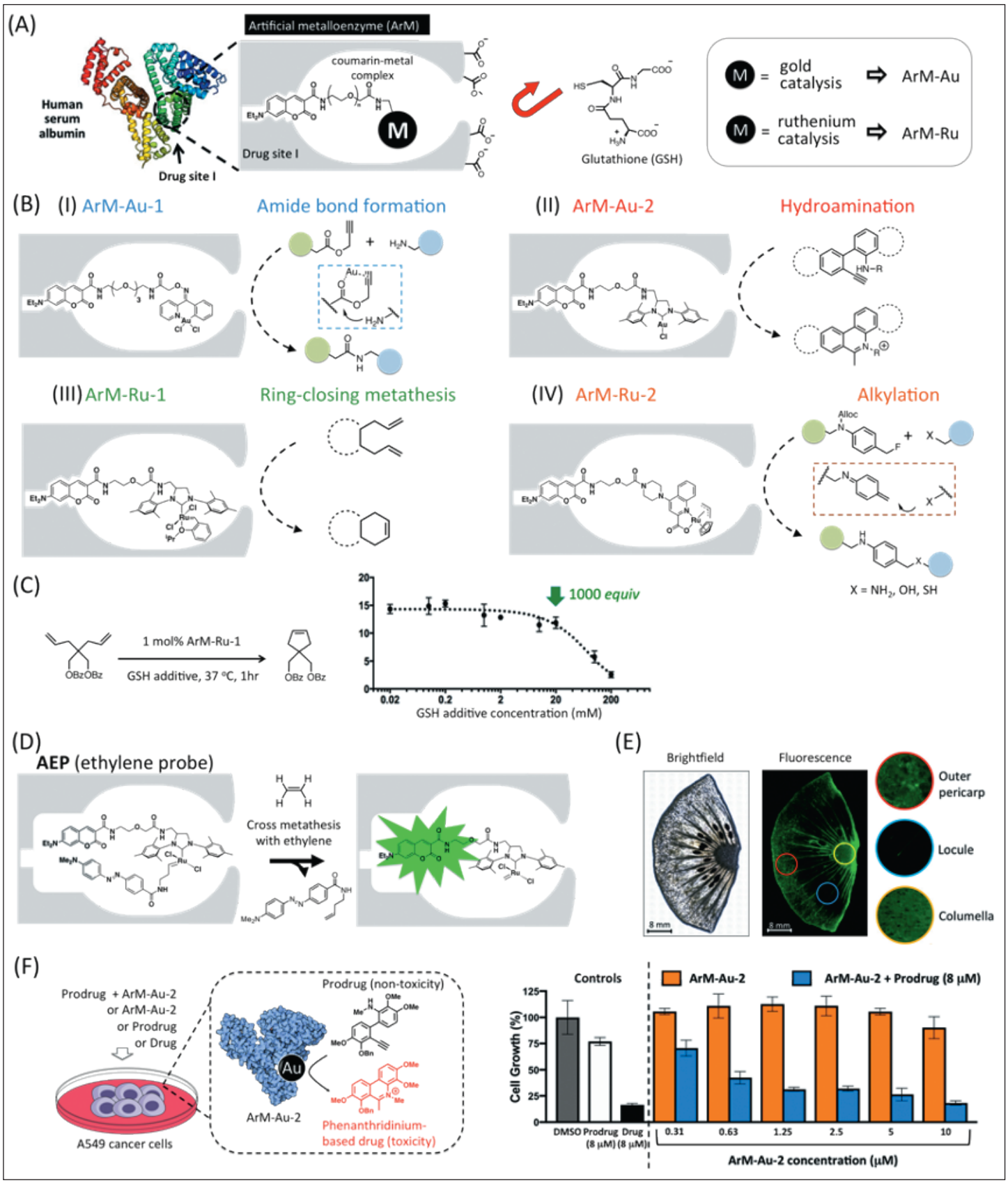

Figure 4. (A) Development of albumin-based artificial metalloenzymes (ArMs) by incubation of metal catalysis into hydrophobic binding pocket (drug site I), and mechanism of glutathione resistance is based on the combined effects of the hydrophobic pocket and surface charge repulsion. (B) The two kinds of ArM-Au can catalyze amide bond formation and hydroamination, and the other two kinds of ArM-Ru can catalyze ring-closing metathesis and alkylation. (C) ArM-Ru-1 was found to be catalytically active even in the presence of up to 1000 x equivalents of glutathione. (D) The mechanistic basis behind the ArM ethylene probe (AEP) relies on the ethylene-triggered release of a quencher. (E) Fluorescent images of ripe kiwifruit slices to highlight the capabilities of AEP to detect endogenously induced ethylene. (F) The ArM-Au-2 successfully triggers drug synthesis to achieve cancer therapy in cell-based assays.

of non-glycosylated protein (HSA) was found to spread out the whole body. Since these are known substrates of receptors normally expressed on nonparenchymal liver cells, our histological studies could indeed confirm that mannose-terminated **1b** was captured by Kupper cells and/or macrophages through the interaction with C-type lectins. Meanwhile, glucosamine-terminated **1a** and hybrid-type **1c** mainly interacted with liver stellate cells and sinusoidal endothelial cells. As for galactose-terminated **1d**, liver accumulation is consistent with being rapidly captured by certain receptors (asialoglycoprtein receptors, ASGPR) expressed on parenchymal hepatocytes. Intriguingly, dissection studies revealed an excretion pathway where **1d** was found to be shuttled to the gall bladder and intestines from liver. On the other hand, sialylated-terminated **1f-g** were

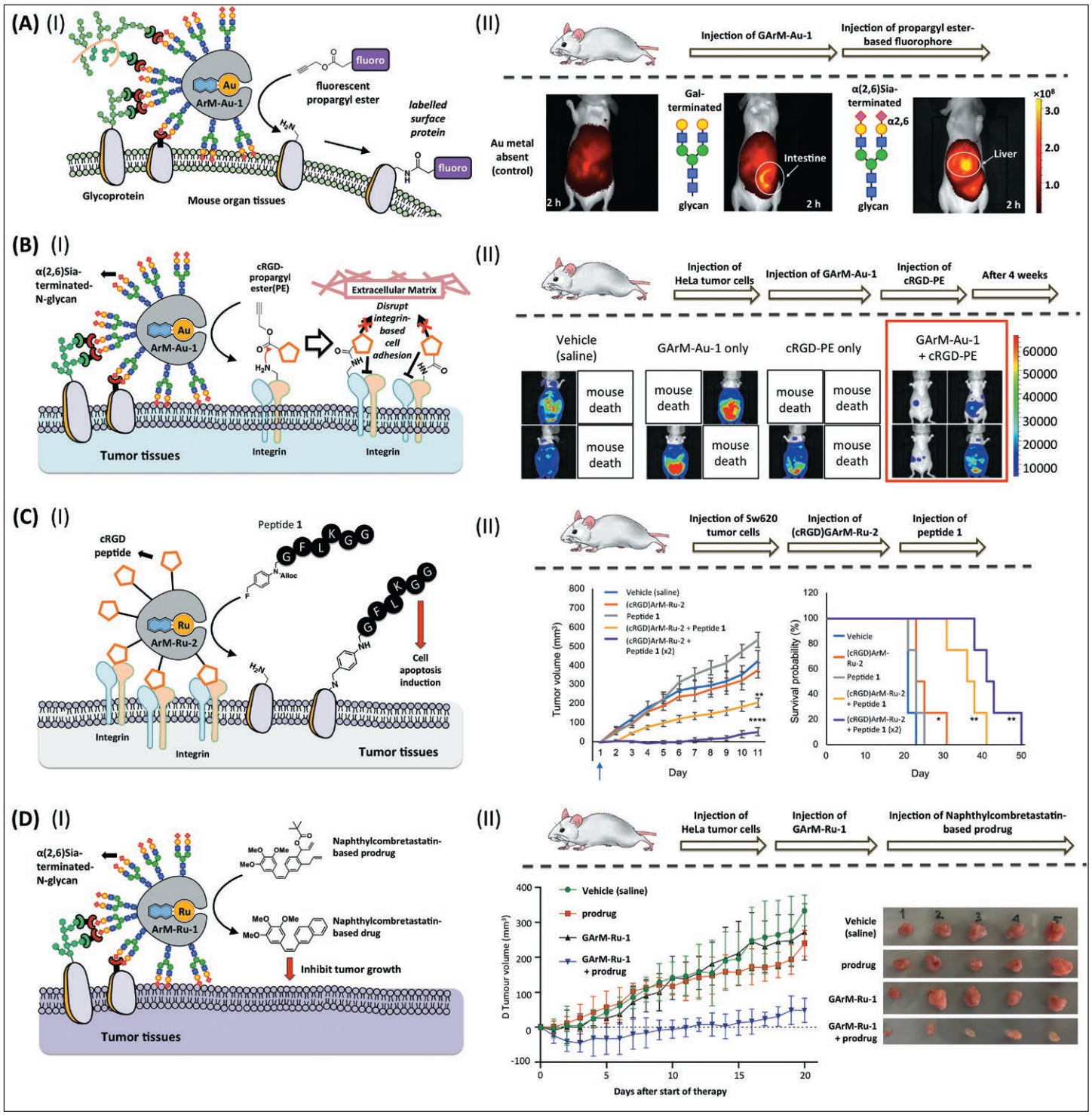

Figure 5. (A) In vivo labeling of organ by the organ-targeting GArM-Au-1. I) Illustration of protein labeling on the surface of targeted cell by the GArM-Au-1 mediated reaction. II) Imaging data displayed that labeling of organs in mice were dependent on the identify of the linked N-glycan. (B) In vivo prevention of tumor onset and progression via SeCT therapy. I) Illustration of labeling target tumor cells with cRGD moieties in vivo to block integrin-based cell adhesion via the GArM-Au-1. II) A representative set of the tumor progression in mice after 4 weeks through IVIS imaging results. (C) In vivo inhibition of tumor growth using a therapeutic peptide via SeCT therapy. I) Illustration of labeling target tumor cells with a therapeutic peptide via (cRGD)ArM-Ru-2. II) Measurement of tumor size in mice over time. Comparison of mice survival rates under various treatment. (D) In vivo synthetic prodrug therapy against HeLa tumour growth in mice. I) Schematic depiction of HeLa targeted activation of prodrug using the GArM-Ru-1. II) Measurements of tumor size (mm3) in mice over time. Tumours were initially implanted in mice and developed over 4 days before therapy.

shuttled towards the kidneys and urinary bladder from liver. To further increase glycan complexity, the biodistribution in mice based on the usage of heterogeneous glycoproteins **1e** and **1h**, containing additional branching, was also studied[25]. Although glycoprotein **1e** followed a pathway similar to **1d**, it showed higher accumulation in intestines compared to **1d**. The glycoprotein **1h** accumulation compared glycoproteins **1f**-**g** was rather directed to the kidneys and then excreted via urinary bladder.

Moving away from biodistribution, we next focused on the investigation of the tumor targeting capacity of artificial glycoproteins. According to the in vivo imaging data (Fig. 3D)[28], homogeneous α(2,3)-sialic acid terminated glycoprotein **1f** was the most promising, as it accumulated at a significantly higher level in the A431 tumor than the other tested glycoproteins **1a**, **c**, **d**, and **g**. With the aim of improving upon this modest target, the focus of a later study was on artificial glycoproteins composed of heterogeneous glycan assemblies. To test differential tumor selectivity, several heterogeneous glycoproteins were injected into mice implanted with 3 different tumors (HeLa, DLD-1, and U87MG).[29] In this study, the most significant observation was that the α(2,3)-sialo and α(2,6)-sialo terminated glycoprotein **2a** showed selective accumulation to HeLa tumors, while it showed no targeting to the DLD-1and U87MG tumors. A possible explanation for this result is that HeLa cells are known to express both galectin-1 and siglec-3 lectins, which are receptors for α(2,3)-sialic acid and α(2,6)-sialic acid, respectively. In contrast, DLD-1 and U87MG only express galectin-1 lectin. Given this promising result, to multiply technique to more selectively target tumors, we developed another approach using higher-order heterogeneous glycoalbumins that were conjugated with four kinds of complex *N*-glycans.[33] As depicted in Figure 3D-(III), the in vivo data revealed that the higher-order glycoalbumin **3b** displayed the strongest targeting towards SW620 tumors than lower-order glycoalbumin **3a**.

Overall, the use of glycan pattern recognition for organs or cancer cells targeting represents a novel and promising strategy for the development of diagnostic, prophylactic, and therapeutic agents for various diseases. Moreover, the use of glycan targeting would have significant advantages over current techniques (i.e. antibody targeting), such as shorter accumulation times and lower immunogenicity.

Biocatalysis and Mild Biocompatible Reactions

Translating abiotic metal catalysts into in vivo synthetic chemistry could encounter numerous challenges regarding their biocompatibility, stability, and reactivity in the complicated biological environment. To solve these issues, we have been actively involved in research related to artificial metalloenzymes (ArMs). ArMs are created by incorporating synthetic metal complexes into protein scaffolds, thereby combining the advantageous features of organometallic and enzymatic catalysts and facilitating the design of novel biocatalysts to perform new-to-nature reactions. To combine with glycan targeting, we were mainly interested in developing ArMs from human serum albumin. Owing to previous direction from the late Prof. Koiji Nakanishi, we decided to utilize coumarin derivatives as an anchor for the hydrophobic binding pocket of albumin (Fig. 4A). With the use of a varying series of coumarin-metal complexes, we developed four kinds of ArMs (mainly ArM-Au and ArM-Ru) (Fig. 4B)[32,36-41]. An important observation from these studies was the discovery that a combination between the deep hydrophobic binding site of albumin and the negatively charged surface of albumin naturally repels entry to hydrophilic metabolites (i.e., glutathione (GSH)). As a result, using a 1,6-heptadiene-based substrate, metastasis activity was shown to proceed even in the presence of up to 1000 x equivalents of GSH additive.[36]

As depicted in Figure 4B-I, we also have developed several biocompatible organic reactions that are applicable to these ArMs. In a preliminary screen to identify amide bond formation, we were surprised to see that the Au(III) complexes coordinated with 2-benzoylpyridine could generate amide via propargyl esters.[32,39,41] Forming an activated ester intermediate (via Au binding) likely leads to amide bond formation via a nucleophile amine. Using this chemistry, we have shown that fluorescent labeling of proteins is possible using propargyl ester-based probes and the ArM-Au-1. On the other hand, the ArM-Au-2 containing Au(I) complexes coordinated with an N-heterocyclic carbene ligand can perform hydroamination to synthesize phenanthridinium derivatives with an excellent turnover number (Fig. 4B-II).[38] In particular, the phenanthridinium moiety has attracted a great deal of attention because of its presence in the scaffolds of several DNA-intercalating agents with antitumor properties. Importantly, hydroamination is not catalyzed by any known naturally occurring enzymes, highlighting the significance of the ArM-Au-2 catalyzed hydroamination under physiological conditions. In addition to that, ring-closing metathesis (RCM) for olefins and ene-ynes can be catalyzed smoothly by the ArM-Ru-1, which incorporates the 2nd generation Hoveyda catalyst.[36,37,41] RCM is widely recognized as a powerful method for creating heterocycles and phenyl moieties that are most significant structural components of pharmaceuticals. Lastly, ArM-Ru-2 can effectively catalyze alkylation with nucleophilic moieties such as thiol, hydroxyl, and amino groups in biomolecules using a benzyl fluoride substrate via a quinone imine intermediate.[40] Overall, the ArM-Au-2 and the ArM-Ru-1 could be utilized as powerful biocatalysts for application in therapeutic in vivo drug synthesis. As for in vivo imaging and drug conjugation, the ArM-Au-1 and the ArM-Ru-2 are competent for these tasks, respectively.

With these new developed biocatalysts and biocompatible reactions, we would like to adapt and apply these technologies for innovative applications. In one of our endeavors, we looked specifically at development of ArM-based biosensors, which would offer a unique path for tailoring against difficult-to-detect metabolites. Ethylene gas is an essential plant hormone that plays a major role in regulating aspects of growth, immunity, and senescence. With this in mind, our group has investigated the creation of an ethylene-sensing ArM biosensor.[37] As depicted in Figure 4D, the basis of this approach is to use the albumin scaffold to solubilize and protect a quenched ruthenium catalyst complex. In the presence of ethylene, cross metathesis is then occurred, leading to the removal of the quencher and the emission of a fluorescent signal. Using the ArM ethylene probe (AEP), the AEP was used to detect changes in ethylene biosynthesis specifically in the outer pericarp of kiwifruit. Since this process is typically unregulated during the ripening process, comparative studies showed an increase in pericarp fluorescence for ripening kiwifruits (Fig. 4E).

Since chemotherapy is not perfectly specific for cancer cells, it has significant side effects on healthy cells. Therefore, another practical application is to employ the ArM-Au-2 as a trigger to control the release of bioactive drugs to improve the defect of chemotherapy.[38] As shown in Figure 4F, the ArM-Au-2 successfully implemented drug synthesis from a non-active prodrug to achieve cancer therapy in a cell-based assay, suggesting the potential of the gold ArM to be a therapeutic ArM for in vivo anticancer application.

Therapeutic in vivo Synthetic Chemistry by GArMs

Given our interest in both glycan targeting and biocatalysis, a natural course of action eventually led to combining both aspects of targeting glycoproteins and ArMs to establish the concept of glycosylated artificial metalloenzymes (GArMs). The ultimate goal of this endeavor will be to eventually establish effective and biocompatible therapeutic ArMs, which can then be conferred with organ/tumor targeting properties by simply decorating the protein scaffold with an appropriate glycan assembly. Our first attempt at developing GArMs came during a study to determine whether specific tissues of mice could be targeted for in vivo labeling (Fig. 5A-I).[32] In this work, glycosylated ArM-Au-1 with the intent to label targeted cells in vivo with propargyl ester-based probes. As depicted in Figure 5A-II, mice were first intravenously injected through the tail vein with a GArM. Then, a near infrared fluorescent propargyl ester (Cy7.5-PE) was injected. As shown in the imaging results, preferential organ labeling could be achieved depending on the identity of the attached glycans; α(2,6)-sialic acid terminated glycans targeted the liver, while Gal terminated glycans targeted the intestines. In the controls, localization of fluorescence labeling was not exhibited in targeted organs.

Given the promising results, more recently, we represented research on selective cell tagging (SeCT) therapy in vivo via the GArM-Au-1 (Fig.

5B).[39] The concept of SeCT therapy is based on a strategy of preferentially tagging specific cells with a biological small molecule. In contrast to traditional chemotherapy that directly eliminates cancer cells using highly cytotoxic drugs, the principal benefit of SeCT therapy allows cancer cells to be tagged using non-toxic chemical moieties that can either disrupt cellular function (ex/ inhibitors of adhesion) or elicit immunological responses (ex/ antigens). Subsequent functional impairment or related biological responses can indirectly lead to cancer cell death without significantly harming surrounding tissue. As depicted in Figure 5B-I, it showed that individual HeLa cancer cells in living mice can be tagged in vivo with cyclic-Arg-Gly-Asp (cRGD) moieties for integrin-blocking, leading to disrupted cell adhesion and compromised successful seeding onto the extracellular matrix (ECM). The mice populations that received just one dosage of the SeCT labeling reagents via intrapenetrial injection showed a significant delay in tumor onset by 4 weeks (Fig. 5B-II), resulting in an improvement in overall survival rates over a period of 81 days.

Following the same concept of the SeCT therapy, we report a cancer therapy based on targeted cell surface tagging with proapoptotic peptide **1** (Ac-GGKLFG-X; X = a benzyl fluoride moiety) that induce apoptosis when attached to the cell surface (Fig. 5C-I).[40] Using the Ru-catalyzed alkylation, the proapoptotic peptide **1** showed excellent therapeutic effects in vivo. In particular, co-treatment with the proapoptotic peptide and the cRGD-coated ArM-Ru-2 significantly and synergistically inhibited tumor growth and prolonged survival rate of tumor-bearing mice after only a single injection (Fig. 5C-II). This is the first report of Ru catalyst application in vivo.

Except of the above samples of therapeutic in vivo synthetic chemistry, we also successfully carried out cancer treatment through localized in vivo drug synthesis. As depicted in Figure 5D, we investigated the design and optimization of synthetic prodrugs that can be robustly transformed in vivo to reach therapeutically relevant levels. To do this, retrosynthetic prodrug design led to the identification of naphthylcombretastatin-based prodrugs, which form highly active cytostatic agents via sequential ring-closing metathesis and aromatization (Fig. 5D-I). Structural adjustments were then made to improve aspects related to catalytic reactivity, intrinsic bioactivity, and hydrolytic stability. Furthermore, in vivo activation by intravenously administered the GArM-Ru-1 was also found to induce significant reduction of implanted tumour growth in mice (Fig. 5D-II).

Conclusions

In an ideal world, one could simply perform reactions developed in a lab setting directly in a living system without any significant loss of reactivity. Unfortunately, for most transition metal catalyzed reactions, the key issues pertaining to biocompatibility are the ease of metal quenching and the intrinsic toxicities of metals. To the credit of many former and current researchers worldwide, numerous ways have been elegantly devised to successfully perform metal-catalyzed reactions in biological settings. Following the success of our works, our group is continuing to research how we can adapt GArMs for biomedical research, as well as to find ways to address the challenges needed for their improvement. Our final ambition is to cure diseases, especially cancer, without any side effects using our technology. Since our technology is targeting, non-invasive, without risk of immunogenicity, non-toxicity, and high efficiency of in vivo drug synthesis, we must point out that our technology, compared to other methods, could be the only possible method to apply to patients for disease treatment in a hospital. We anticipate that our technology will make a substantial contribution to biomedical fields in the future. ◆

References

1. E. Petru, D. Schmahl, Neoplasma 1991, 38, 147.
2. J. Yao, J. Feng, J. Chen, Asain J. Pharm. Sci. 2016, 11, 585.
3. X. Ji, Z. Pan, B. Yu, L. K. De La Cruz, Y. Zheng, B. Ke, B. Wang, Chem. Soc. Rev. 2019, 48, 1077.
4. S. J. Sonawane, R. S. Kalhapure, T. Gocender, Eur. J. Pharm. Sci. 2017, 99, 45.
5. D. C. Luther, R. Huang, T. Jeon, X. Zhang, Y.W. Lee, H. Nagaraj, V. M. Rotello, Adv. Drug. Delivery Rev. 2020, 156, 188.
6. F. Danhier, J. Control. Release 2016, 244, 108-121.
7. A. B. Sengul, E. Asmatulu, Environ. Chem. Lett. 2020, 18, 1659.
8. K. Tanaka, H. Mori, M. Yamamoto, S. Katsumura, J. Org. Chem. 2001, 66, 3099.
9. K. Tanaka, K. Fukase, S. Katsumura, Chem. Rec. 2010, 10, 119.
10. K. Tanaka, K. Fukase, S. Katsumura, Synlett 2011, 2115.
11. K. Fujiki, K. Tanaka, in Encyclopedia of Reagents for Organic Synthesis, John Wiley & Sons, Ltd., 2020, doi: 10.1002/047084289X.rn02050
12. K. Tanaka, T. Masuyama, K. Hasegawa, T. Tahara, H. Mizuma, Y. Wada, Y. Watanabe, K. Fukase, Angew. Chem. Int. Ed. 2008, 47, 102.
13. K. Tanaka, K. Minmi, T. Tahara, Y. Fujii, E. R. O. Siwu, S.Nozaki, H. Onoe, S. Yokoi, K. Koyama, Y. Watanabe, K. Fukase, ChemMedChem 2010, 5, 841.
14. K. Tanaka, Y. Fujii, K. Fukase, ChemBioChem 2008, 9, 2392.
15. K. Tanaka, M. Kitadani, K. Fukase, Org. Biomol. Chem. 2011, 9, 5346.
16. K. Tanaka, K. Moriwaki, S. Yokoi, K. Koyama, E. Miyoshi, K. Fukase, Bioorg. Med. Chem. 2013, 21, 1074.
17. K. Tanaka, S. Yokoi, K. Morimoto, T. Iwata, Y. Nakamoto, K. Nakayama, K. Koyama, T. Fujiwara, K. Fukase, Bioorg. Med. Chem. 2012, 20, 1865.
18. K. Tanaka, E. R. O. Siwu, K. Minami, K. Hasegawa, S.Nozaki, Y. Kanayama, K. Koyama, W. C. Chen, J. C. Paulson, Y. Watanabe, K. Fukase, Angew. Chem. Int. Ed. 2010, 49, 8195.
19. K. Tanaka, K. Minami, T. Tahara, E. R. O. Siwu, K. Koyama, S. Nozaki, H. Onoe, Y. Watanabe, K. Fukase, J. Carbohydr. Chem. 2010, 29, 118.
20. K. Fujiki, S. Yano, T. Ito, Y. Kumagai, Y. Murakami, O. Kamigaito, H. Haba, K. Tanaka, Sci. Rep. 2017, 7, 1912.
21. K. Fujiki, Y. Kanayama, S. Yano, N. Sato, T. Yokokita, P. Ahmadi, Y. Watanabe, H. Haba, K. Tanaka, Chem. Sci. 2019, 10,1936.
22. K. Tanaka, Y. Nakamoto, E. R. O. Siwu, A. R. Pradipta, K. Morimoto, T. Fujiwara, S. Yoshida, T. Hosoya, Y. Tamura, G. Hirai, M. Sodeoka, K. Fukase, Org. Biomol. Chem. 2013, 11, 7326.
23. A. Ogura, K. Tanaka, Tetrahedron 2015, 71, 4518.
24. K. Tanaka, M. Kitadani, A. Tsutsui, A. R. Pradipta, R. Imamaki, S. Kitazume, N. Taniguchi, K. Fukase, Org. Biomol. Chem. 2014, 12, 1412.
25. L. Latypova, R. Sibgatullina, A. Ogura, K. Fujiki, A. Khabibrakhmanova, T. Tahara, S. Nozaki, S. Urano, K. Tsubokura, H. Onoe, Y. Watanabe, A. Kurbangalieva, K. Tanaka, Adv. Sci. 2017, 4, 1600394.
26. R. Sibgatullina, K. Fujiki, T. Murase, T. Yamamoto, T. Shimoda, A. Kurbangalieva, K. Tanaka, Tetrahedron Lett. 2017, 58, 1929.
27. A. Ogura, T. Tahara, S. Nozaki, K. Morimoto, Y. Kizuka, S. Kitazume, M. Hara, S. Kojima, H. Onoe, A. Kurbangalieva, N. Taniguchi, Y. Watanabe, K. Tanaka, Sci. Rep. 2016, 6, 21797.
28. A. Ogura, T. Tahara, S. Nozaki, H. Onoe, A. Kurbangalieva, Y. Watanabe, K. Tanaka, Bioorg. Med. Chem. Lett. 2016, 26, 2251.
29. A. Ogura, S. Urano, T. Tahara, S. Nozaki, R. Sibgatullina, K. Vong, T. Suzuki, N. Dohmae, A. Kurbangalieva, Y. Watanabe, K. Tanaka, Chem. Comm. 2018, 54, 8693.
30. Y. Nakamoto, A. R. Pradipta, H. Mukai, M. Zouda, Y. Watanabe, A. Kurbangalieva, P. Ahmadi, Y. Manabe, K. Fukase, K. Tanaka, ChemBioChem 2018, 19, 2055.
31. K. Nakamura, K. Tsubokura, A. Kurbangalieva, Y. Nakao, T. Murase, T. Shimoda, K. Tanaka, J. Carbohydr. Chem. 2019, 38, 127.
32. K. Tsubokura, K. K. H. Vong, A. R. Pradipta, A. Ogura, S. Urano, T. Tahara, S. Nozaki, H. Onoe, Y. Nakao, R. Sibgatullina, A. Kurbangalieva, Y. Watanabe, K. Tanaka, Angew. Chem., Int. Ed. 2017, 56, 3579.
33. I. Smirnov, R. Sibgatullina, S. Urano, T. tahara, P. Ahmadi, Y. Watanabe, A. R. Pradipta, A. Kurbangalieva, K. Tanaka, Small, 2020, 16, 2004831.
34. I. Smirnov, I. Nasibullin, A. Kurbangalieva, K. Tanaka, Tetrahedron Lett., 2021, 72, 153089.
35. S. Ihara, E. Miyoshi, J. H. Ko, K. Murata, S. Nakahara, K. Honke, R. B. Dickson, C. Y. Lin, N. Taniguchi, J. Biol. Chem. 2002, 277, 16960.
36. S. Eda, I. Nasibullin, K. Vong, N. Kudo, M. Yoshida, A. Kurbangalieva, K. Tanaka, Nat. Catal. 2019, 2, 780.
37. K. Vong, S. Eda, Y. Kadota, I. Nasibullin, T. Wakatake, S. Yokoshima, K. Shirasu, K. Tanaka, Nature Commun. 2019, 10, 5746.
38. T.-C. Chang, K. Vong, T. Yamamoto, K. Tanaka, Angew. Chem. Int. Ed. 2021, 60, 12446.
39. K. Vong, T. Tahara, S. Urano, I. Nasibullin, K. Tsubokura, Y. Nakao, A. Kurbangalieva, H. Onoe, Y. Watanabe, K. Tanaka, Sci. Adv. 2021, 7, eabg4038.
40. P. Ahmadi, K. Muguruma, T.-C. Chang, S. Tamura, K. Tsubokura, Y. Egawa, T. Suzuki, N. Dohmae, Y. Nakao, K. Tanaka, Chem. Sci. 2021, 12, 12266-12273.
41. Y. Lin, K. Vong, K. Matsuoka, K. Tanaka, Chem. Eur. J. 2018, 24, 10595.
42. Nasibullin, I. Smirnov, P. Ahmadi, K. Vong, A. Kurbangalieva, K. Tanaka, Nature Commun. DOI : 10.1038/s41467-021-27804-5.