Annual Report 2016

Volume 1 1

Kagawa School of Pharmaceutical Sciences

Tokushima Bunri University

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Preface to the tenth issue of *The Annual Report*

The Faculty of Pharmaceutical Sciences at the Kagawa Campus was founded in April, 2004. Our purpose is to educate students to become professional pharmacists and basic scientists with sufficient knowledge of chemistry, biology, and pharmaceutical thought to practice with humanity and a high sense of ethics and social responsibility. In April, 2005, the Kagawa School of Pharmaceutical Sciences was established with masters and doctoral programs. The undergraduate course of the Kagawa School of Pharmaceutical Sciences was reorganized in April, 2006, into two departments, the Department of Pharmacy (with a six-year training program) and Pharmaceutical Sciences (with a four-year training program). The mission of the Department of Pharmacy (the six-year program) is to train highly qualified pharmacists who are knowledgeable about advanced medical treatment, and who also can contribute to the prevention and treatment of diseases. Society's view of pharmacy education has changed, not only in medical fields but also in other fields, to the fostering of a healthy and safe society. In order to fulfill this mandate, the Department of Pharmaceutical Sciences (the four-year program) educates students to become key members of society who can play an active part in all social fields with their broad background in pharmacy, and who can become outstanding life scientists by making signal contributions to basic science. But Kagawa School of Pharmaceutical Sciences has turned into only the program of the Department of Pharmacy in April, 2012, in order to match the needs of the region. Our faculty, which has cutting-edge equipment, succeeds the principles at the foundation and has trained professionals with a research mind who can respond the social demands, and a variety of human resources who can contribute to the promotion of health of the people.

Guided by these principles, we laid the foundation for the Kagawa School of Pharmaceutical Sciences and dedicated ourselves to this effort. We established Institute of Neuroscience in the Kagawa School of Pharmaceutical Sciences, and the Institute of Neuroscience was reorganized and has now been expanded into four divisions in April, 2007. We published the first issue of The Annual Report of the Kagawa School of Pharmaceutical Sciences, highlighting our educational, research, management, and philanthropic achievements, by looking back upon the past three years of our activities, in 2006. We are now publishing the eleventh issue of the Annual Report by looking back upon the past five years of our activities. I would like all of the staff to utilize these issues to perform a self-assessment and to look forward to the future development of their activities. In addition, I expect that this issue will assist our staff in expanding both intramural and extramural interdisciplinary exchanges.

The Kagawa School of Pharmaceutical Sciences is always making progress on the road toward realizing our goals. I would be very grateful if all the people who read this annual report would kindly give me their opinions and/or comments with a view toward continuing to improve the Kagawa School of Pharmaceutical Sciences.

Hiroshi Miyazawa, Ph.D., Dean
Kagawa School of Pharmaceutical Sciences
Tokushima Bunri University

Contents

A Short History of *The Kagawa School of Pharmaceutical Sciences*

1895 Sai Murasaki founds a private vocational school in the city of Tokushima.

1966 Tokushima Women's University is founded.
Faculty of Home Economics admits its first students.

1968 Faculty of Music admits its first students.

1972 Tokushima Women's University is renamed Tokushima Bunri University.
Faculty of Pharmaceutical Sciences admits its first students.

1983 The Kagawa Campus of Tokushima Bunri University opens.
Faculty of Literature admits its first students on the Kagawa campus.

1989 Faculty of Engineering admits its first students on the Kagawa campus.

1995 The 100th anniversary of the founding of the Murasaki Gakuen.

2000 Faculty of Policy Studies admits its first students.

2004 Faculty of Pharmaceutical Sciences at Kagawa Campus admits its first students on the Kagawa campus; the Department of Pharmaceutical Technology opens 10 laboratories.
The rooms for teaching staff open on the fourth floor of the Research and Media Library.
The office of the Faculty of Pharmaceutical Sciences at Kagawa Campus opens on the first floor of the Lecture Building.

2005 The Kagawa School of Pharmaceutical Sciences is established on the Kagawa campus.
Masters and doctoral programs begin at the Kagawa School of Pharmaceutical Sciences.
Pharmaceutical Sciences Research and Laboratory Buildings are completed.
The Center for Instrumental Analysis opens in the Pharmaceutical Sciences Research Building.
The Center for Radioisotope and Laboratory Animals opens in the Pharmaceutical Sciences Laboratory Building.
The Medicinal Plant Garden is established.
The 110th anniversary of the founding of the Murasaki Gakuen.

2006 The six-year undergraduate program in the Department of Pharmacy, Kagawa School of Pharmaceutical Sciences accepts its first students.
The four-year undergraduate program in the Department of Pharmaceutical Sciences, Kagawa School of Pharmaceutical Sciences accepts its first students.
A mock pharmacy opens in the Lecture Building for student training.
The construction of eighteen laboratories and one research institute for the Kagawa School of Pharmaceutical Sciences is completed.
SENKA Endowed Chair for "Practical Drug Discovery and Development" was established.

2007 The research institute (Institute of Neuroscience) was reorganized and expanded into four divisions.

2012 The four-year Graduate School of Pharmaceutical Sciences (Department of Pharmaceutical Sciences Doctoral program) was installed.

2014 The 10th anniversary of the foundation of Kagawa School of Pharmaceutical Sciences

2015 The 120th anniversary of the founding of the Murasaki Gakuen.

Kagawa School of Pharmaceutical Sciences
Tokushima Bunri University

Laboratory for Analytical Chemistry

Staff

Professor: Kentaro Yamaguchi, Ph. D (Apr. 2004)
Educational History:
Graduated from University of Electrocommunications in Mar. 1975
Ph. D (Tokyo University)
Latest Work Record:
Associate Professor in Chiba University

Associate Professor: Masahide Tominaga, Ph.D.
Educational History:
D. Eng. University of Tokyo, 2000

Lecturer: Masatoshi Kawahata, Ph. D
Educational History:
Graduated from Graduate School of Tokushima Bunri University in Mar. 2006

Research

Laboratory of Analytical chemistry has designated that it contributes to pharmaceutical technology with analyzing molecular structures, physical properties, chemical reactivity, and functions. The connection between dynamic behavior of biomolecules and its reactivity-function relationship as well as the detection of unstable reaction intermediate in solution are our major subjects. We have been engaged in the study to produce new analysis method, which reveals the generating mechanism of the outstanding functionalities and the physical properties of the materials in solution based on microanalysis in the atomic resolution. We also develop the analytical and chemical technique in solution that is based on quantum mechanics and statistical mechanics. These results might be applied to pharmaceutical organic synthesis.

Development in the field of mass spectrometry:

In the laboratory, we have succeeded in the development of the 'Cold-Spray Ionization (CSI)' method. CSI is designed for mass spectrometric detection of labile organic species. It may be an appropriate method to analyze in solution the structures of biomolecular complexes, labile organic species including Grignard reagents, asymmetric catalysis, and supermolecules. The method, a variant of Electrospray ionization (ESI)-MS, operates at low temperature, allow simple and precise characterization of labile non-covalent complexes that are difficult or impossible to observe by conventional MS techniques, including fast atom bombarment (FAB), and matrix assisted laser desorption ionization (MALDI), as well as ESI.

Biopolymer analysis:

The behavior of important in vivo molecule such as protein, nucleic acid, and lipid, will be made clear by using our newly developed method CSI mass spectrometry. The structure of multistranded DNA of duplex, triplex and quadruplex DNA have been examined by electrospray ionization (ESI) MS. However, non-covalent complexes of multiply stranded DNA are difficult to observe by conventional methods, because of their low melting temperature (T*m*). The characterization of triple- and quadruple-stranded oligodeoxynucleotides was carried out by means of CSI-MS. In consequence, it is proved that DNA has been made clear to take hyper-stranded structures combining various multiply stranded helical components.

Analysis of organic reaction mechanism:

A new CSI mass spectrometric procedure RTS (reaction tracking system), which can trace the time-dependent simultaneous change of the raw materials, products and inter mediates in an organic reaction under CSI condition has been developed. It would be expected that behavior of each component becomes clear and that the appropriate design become possible to make the reaction more efficiently.

Observation of the giant molecules by means of mass spectrometry:

Mass Spectrometry (MS) has been developed and adopted to wide variety of analytical chemistry in recent years.

Although MS was basically developed for high molecular weight substances in the field of biochemistry, the measurement of huge molecules over 10k Da is still very difficult. This is caused by the ionizing problems, stability of the compounds and the existence of various impurities.

We develop some new techniques to overcome these problems by using newly equipped FT-ICR mass spectrometer.

Construction of Adamantane-Based Macrocycles and Cages:

Synthetic macrocyclic compounds are considerably important receptor molecules, and they provide an opportunity to interact with various guest molecules by the binding sites of multiple functional groups and well-defined cavities within their frameworks. Adamantane derivatives are unique and specific compounds, because they are mechanically rigid and conformationally well-defined. However, the design and synthesis of adamantane-based host molecules and their applications in host-guest systems remain largely unexplored. We reported the construction and structural analysis of various types of nano-sized adamantane-based macrocycles and cages bearing rectangle, square, and spherical shapes. Their macrocycles encapsulate electron-poor guest molecule, 1,3,5-trinitrobenzene via charge-transfer interactions. Further, adamantane-based macrocycles showed the generation of unique molecular networks in the solid state. The adamantane-based macrocycles formed tubular structures with channels, which were assembled into three-dimensional networks through multiple intermolecular interactions.

Self-assembled Nano-scale Structures Using Macrocyclic Framework as Molecular Block:

Recently, several functionalized macrocycles were utilized as building blocks for self-assembled nanostructures including vesicles, tubes, fibers, and others. We synthesized a series of oxacyclophanes, azacyclophanes, and salen-based macrocycles bearing adamantane parts. By utilizing the specific properties of spherical shape, bulky skeleton, and lipophilicity of the adamantane derivatives, these adamantane-derived macrocycles were induced into hollow spherical aggregates with a multilayer membrane in organic and aqueous solutions. The hollow spheres were induced into fibrous and/or network aggregates, which were eventually transformed into single crystals. These results afforded the evidences for a morphological change and phase transition occurring from the solution into a solid.

Publications (2012~2017)

[Original papers]

2017

1. Ning, Y.; Fukuda, T.; Ikeda, H.; Otani, Y.; Kawahata, M.; Yamaguchi, K.; *Ohwada, T. (2017). Revisiting secondary interactions in neighboring group participation, exemplified by reactivity changes of iminylium intermediates. Org. Biomol. Chem., 2017, 15, 1381-1392.

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3. *Sasaki, M.; Ando, M.; Kawahata, M.; Yamaguchi, K.; Takeda, K. (2016). Spontaneous Oxygenation of Siloxy-*N*-silylketenimines to α-Ketoamides. Org. Lett., 2016, 18, 1598-1601.
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with a Dinuclear Co^{III}–Pyridylmethylamine Complex. Inorg. Chem., 55, 1154-1164.

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Adamantane-Based Macrocycles. Tetrahedron, 70, 2576-2581.

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Research

Molecular and neural mechanisms of eyeblink classical conditioning

Introduction: Associative learning is a fundamental form of cognition in humans and animals. Eyeblink classical conditioning (EBCC) is a form of associative learning that has been most extensively studied at the neurological and behavioral level. Its basic neural circuitry and neural mechanisms have been demonstrated to be similar in all mammals. Since the same paradigm is applicable to humans as well as non-human mammals, there is growing interest in EBCC for the study of human diseases of motor and memory impairment, in parallel with detailed studies of the molecular and neural mechanisms in animal models.

Typical EBCC experiments use a tone as the conditioned stimulus (CS), and a periorbital shock or corneal air puff as the unconditioned stimulus (US). By repeated presentations of the CS paired with the US, the CS comes to elicit an eyeblink, which is called the conditioned response (CR). Previous studies have indicated that the cerebellum and brainstem are sufficient for *delay* conditioning in which the US is delayed and co-terminates with the CS. On the other hand, *trace* conditioning, in which a stimulus-free trace interval intervenes between the CS and US, requires other brain regions, including the hippocampus and the medial prefrontal cortex (mPFC).

Basic Functions of the Cerebellum and Brainstem: To investigate the basic properties of the essential neural circuits in the cerebellum and brainstem, we have developed a decerebrate guinea pig preparation, in which a section is made between the thalamus and the superior colliculus and all of the brain tissue above the section is aspirated. Decerebrate animals readily acquire the CR in delay conditioning. When a longer tone CS is used, the learning becomes slower. These CRs are adaptive and appropriately timed relative to the US. Subsequent CS-alone trials cause extinction of the CR. These characteristics of eyeblink conditioning are similar to those reported previously in intact animals of various species, suggesting that the cerebellum and brainstem are sufficient for this type of learning.

We also study trace eyeblink conditioning in decerebrate guinea pigs. A 350-ms tone CS is paired with a 100-ms periorbital shock US with a trace interval of either 0, 100, 250, or 500 ms. Decerebrate animals readily acquire the CR with a trace interval of 0 or 100 ms. Even in the paradigm with a 500-ms trace interval, which is known to depend critically on the hippocampus in all animal species examined, the decerebrate guinea pigs acquire the CR, with the adaptive timing seen in the other paradigms with a shorter trace interval. However, it takes many more trials to learn when we employ the 500-ms trace paradigm rather than the shorter trace-interval paradigms, and the CR expression is unstable from trial to trial. When decerebrate animals are conditioned step by step with a trace interval of 100, 250, and 500 ms (in that order), they easily acquire the adaptive CR with the 500-ms trace interval. However, the CR% decreases after the trace interval is shifted from 250 ms to 500 ms, a decrease that is not observed with the shift from 100 ms to 250 ms. These results suggest that the cerebellum and brainstem can maintain the “trace” of the CS and associate it with the US even in the 500-ms trace paradigm, but that the forebrain might be required to facilitate the association and stabilization of the memory.

Cerebellar Cortical Mechanism of EBCC: Long-term depression (LTD) at parallel fiber-Purkinje cell synapses in the cerebellar cortex has been proposed as the neural substrate for EBCC. Since the glutamate receptor subunit δ2 (GluRδ2) is selectively expressed at the dendritic spines of the Purkinje Cell (PC) and is essential for the induction of cerebellar LTD, GluRδ2-null mice (in which cerebellar LTD is specifically impaired) provide a useful means to test the cerebellar LTD hypothesis. Mutant mice lacking GluRδ2 show severe learning impairment in *delay* conditioning, but learn normally

in *trace* conditioning. This surprising finding has now been confirmed in experiments with another line of mutant mice lacking phospholipase Cβ4 and with wild-type mice subjected to intracerebellar injection of the NO synthase inhibitor L-NAME, both of which lack cerebellar LTD. Therefore, there may be variations in the cerebellar neural substrates for eyeblink conditioning, depending on the CS-US temporal overlap.

We have recently found that the muscarinic acetylcholine receptor antagonist scopolamine and the NMDA receptor antagonist MK-801 impair learning in trace conditioning experiments with a zero trace-interval (trace 0 paradigm experiments) in GluRδ2$^{-/-}$ mice, and that the metabotropic glutamate receptor subtype 1 outside the cerebellum is essential for trace conditioning but not for delay conditioning. These findings suggest a contribution of the hippocampus to the LTD-independent learning mechanism. To examine this possibility further, we have looked at the effects of hippocampal lesions on learning in GluRδ2$^{-/-}$ mice. GluRδ2$^{-/-}$ mice whose dorsal hippocampi were aspirated exhibit severe learning impairment in the trace 0 paradigm experiments, while control GluRδ2$^{-/-}$ mice that received a lesion in the cortex overlying the hippocampus are able to learn promptly. Wild-type mice do not show such hippocampal dependency in the trace 0 paradigm. We therefore concluded that the hippocampus is essential for learning with a trace 0 paradigm when cerebellar LTD is disrupted. In contrast, GluRδ2$^{-/-}$ mice that receive post-training hippocampal lesions retain the memory as well as the control GluRδ2$^{-/-}$ mice do. The hippocampal lesion also does not affect memory retention in wild-type mice. These results suggest that the hippocampus is *not* essential for *retention* of motor memory with a trace 0 paradigm in LTD-deficient mice. Thus, the present studies clearly argue that the hippocampus is essential for memory formation in cerebellar motor learning when cerebellar LTD is disrupted. These studies suggest that the neural network which underlies learning and memory is both flexible and robust.

Reorganization of Brain Circuitry during Memory Consolidation: Previous studies, including those described above, have confirmed the time-limited involvement of the hippocampus in mnemonic processes and have suggested that there is reorganization of the responsible brain circuitry during memory consolidation. For temporal characterization of such reorganization, we carried out EBCC experiments with a trace interval of 500 ms in rats with ablation of one of three brain regions: the hippocampus, the mPFC, or the cerebellum. At various time intervals after establishing the trace conditioned response (CR), rats receive an aspiration of one of these regions. After recovery, the animals are tested for their retention of the CR. When ablated one day after the learning, both the hippocampal lesion and the cerebellar lesion groups exhibit severe impairment in their retention of the CR, whereas the mPFC lesion group show only a small decline. As we increase the interval between the lesion and the learning, the effect of the hippocampal lesion decreases and that of the mPFC lesion increases. When ablated 4 weeks after the learning, the hippocampal lesion group exhibits CRs that are as robust as those of the corresponding control group. In contrast, the mPFC lesion and cerebellar lesion groups fail to retain the CRs. These results indicate that the hippocampus and the cerebellum, but only marginally the mPFC, constitute a brain circuitry that mediates recently acquired memory. As time elapses, the circuitry is reorganized to use mainly the mPFC and the cerebellum, but not the hippocampus, for remotely acquired memory.

Application of EBCC to Studies of Human Memory Loss: In addition to its great advantage as a model system for learning and memory, EBCC is expected to have wide potential application to clinical studies, including motor impairment (e.g. Parkinson's disease), dementia (e.g. Alzheimer's disease), and other psychopathologies. Such studies using mouse models of human memory loss (experiments using aged animals, senescence- accelerated SAMP8 mice, obese mice and animals injected with β-amyloid peptide) are currently under way.

The Study of the structure and the dynamics of proteins by means of site-directed spin-labeling ESR

The spin label reagent attached to cysteine residue tells us its environment and the structural information of the protein through the ESR spectrum. In other words, the spin label acts as a reporter. In the case of two spin labels introduced into a protein, we can get the distance between two labels by the spectrum. The merit of this method is that we can monitor only the spin label whatever the sample condition is. So we can measure the membrane protein in lipid for example, that is difficult to measure by other spectroscopic method.

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Research

We conduct chemical and biological research on the components of medicinal plants and crude drugs in order to advance the development of therapeutic pharmaceuticals. The lab focuses on determining the structure of potential compounds, the relationship between that structure and their biological activity, and the role of genes in the biosynthesis of compounds and their function in human physiology. In addition, new avenues in systems biology and metabolomics are being explored and there is ongoing research on the prevention of illegal drug circulation and use, and on the evaluation of drug quality.

I. *Research on the biological active chemical constituents of health foods*

We found that royal jelly acted at the early stages of the G_1 phase and the S phase of a cell cycle and controlled multiplication of human osteosarcoma cell line, MG-63 cell. Separation of the active water extract by a dialysis membrane and a solid phase extraction suggested that active substances were high polar low molecular compounds. Furthermore, the existence of nitrogen-containing compounds having acidic groups was suggested by LC/MS (ESI^-) analyses. Further isolation procedure identified that the main active component was AMP N_1-oxide. Continuous examination revealed also the existence of adenosine N_1-oxide, ADP N_1-oxide, ATP N_1-oxide, and NAD N_1-oxide as active ingredients. AMP N_1-oxide and adenosine N_1-oxide inhibited multiplication of MG-63 cell strongly, and their control of the G_1 to S phase comparing with AMP as 1/100 low concentration was found. From these facts, AMP N_1-oxide, adenosine N_1-oxide, and other N_1-oxide are considered to be the main ingredients that contribute at MG-63 cell-growth control of royal jelly.

II. *The search of the Alzheimer therapeutic drug from natural resources*

Donepezil hydrochroloride of the choline esterase inhibitor is used as Dementia and Alzheimer therapeutic drag. Galantamine, the metabolite of the Amaryllidaceae plant acts in similar mechanism, and approval in the country is examined. In addition, Kampo medicine nominates an effect for condition improvement. We perform construction pro-screening to search for the therapeutic drug from a crude drug and a medical plant.

III. *The study of anti-Leishmaniasis therapeutics*

Leishmaniasis is a parasitic disease caused by species of the genus Leishmania. Over 20 of which are known to be pathogenic to humans、and the disease is endemic in some tropical and subtropical regions of the world. *Leishmaniasis* is transmitted by small biting sandflies (*Phlebotomus* spp.), causing a disease which currently afflicts twelve million people in 88 countries. *Leishmania major,* the causative agent of cutaneous leishmaniasis, is a digenetic parasite that exists as an extracellular promastigote within the insect vector (sandfly), and as a nonmotile intracellular amastigote within the phagolysosome of macrophages and other cells of the reticuloendothelial system of the mammalian host. Treatment options for leishmaniases include pentavalent antimonials as first-line drugs, and amphotericin B and pentamidine as second-line drugs. However, these drugs are extremely toxic and usually too expensive for general use, and more economical and less toxic drugs are thus being sought. We have been searching for plant compounds that are active against *Leishmania major*, *L. panamensis, L. guyanensis, and L. peruviana*, exhibited significant activity against the pathogenic protozoan, and newly assay method. Recently, we isolated leishmanicidal naphthoquinones from *Tectona grandis*.

IV. *Research on the chemical components of illegal drugs of plant origin*

1. Khat is a fresh leaf of evergreen shrub *Catha edulis* (Celastaceae) that grows naturally or is grown in Ethiopia, East and Southern Africa, and Yemen, etc., and a lot of people in Africa and Arabia nations use this leaf traditionally as a stimulant biting, and, as a result, it is assumed to obtain the feeling of well-being at the same time as hungry and tiredness's softening. The stimulating component of Khat was believed to be *d*-norpseudoephedrine until cathinone was identified as a main active constituent at the end of 1970's. This cathinone is regulated as narcotics and psychotropic drug, and there is an action similar to (+)-amphetamine that is the synthetic central nervous system stimulation medicine and the strength is assumed to be this level. I synthesized cathinone and ephedrine as an authentic sample to use for the analysis of the drug.

2. *Salvia divinorum* which belongs to Labiatae family is used in traditional spiritual and curative practices by the indigenous Mazatec people of southern Mexico. Salvinorin A (Sal A), which is the neoclerodane ditrepene and is an extremely potent and highly selective kappa opioid receptor agonist, is the main active constituent isolated from the leaves of *S. divinorum.* The sale of *S. divinorum* has become prohibited due to its psychoactive effect in Japan in recent years. The main objectives of this research are to develop immunoassays using anti-Sal A monoclonal antibody (MAb). The icELISA, which has a measuring range from 0.156 μg/ml to 1.25 μg/ml for Sal A, was developed to distinguish *S. divinorum* among various Labiatae plants. In addition, we are preparing the immunochromatographic strip to realize much more rapid analysis. These immunoassays using anti-Sal A must be a convenient authentification method for *S. divinorum* samples.

V. *Research on Development of Preparative Separation Method of Biologically Active Natural Products by Centrifugal Partition Chromatography : Preparative separation of lancemaside A from Codonopsis lanceolata by CPC*

The roots of *Codonopsis* sp. (Campanulaceae) have been used in folk medicine in China, Korea, and Japan for the treatment of bronchitis, cough, spasm, and inflammation. Recently, it was demonstrated that a hot water extract of *C. lanceolata* roots promoted spermatogenesis and improved sexual motion. Moreover, three phenylpropanoids were identified as the active compounds that promoted spermatogenesis, while several saponins, including lancemaside A, were isolated, and lancemaside A was identified as the active compound that improves sexual motion. Although it is assumed that *C. lanceolata* roots are highly safe since they have been used for a long time, general and specific toxicity tests for safety assurance of the active integrants are required. In general, a large amount of purified compounds is required to assess the effectiveness and to perform safety tests. Therefore we attempted to develop a simple and efficient method for the preparative isolation of lancemaside A from the hot water extract of *C. lanceolata* roots, and resulted in the successful preparative separation of lancemaside A along with two other saponins from the saponin fraction of *C. lanceolata* by CPC.

Publications (2012.4~2017.3)

[Original papers]

2016

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7. Mori-Yasumoto, K., Izumoto, R., Fuchino, H., Ooi, T., Agatsuma, Y., Satake, M., Sekita, S. (2012). Leishmanicidal activities and cytotoxicities of bisnaphthoquinone analogues and naphthol derivatives from Burman *Diospyros burmanica*, Bioorganic and Medicinal Chemistry 20, 5215-5219.

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Research

Design and synthesis of the novel vitamin D receptor ligands

We have been interested in functions of the nuclear receptors modulated by small molecules, which can be critical to certain disease states. In particular, novel analogues targeted to vitamin D receptor (VDR) were designed and synthesized to understand how the subtype-free, singular VDR can deliver the diverse biological activities vitamin D, as well as to allow the development of potential therapeutic agents with selective activity profiles for the treatment of cancer or osteoporosis. Syntheses of the analogues were carried out by a convergent method using a palladium catalyst. Separate preparation of the requisite A-ring enyne precursors has developed from the 3-buten-1-ol derivatives. Modification in the A-ring, as well as in the side chain of vitamin D, resulted in exceptionally potent compounds with unique activity profiles.

Publications (2012~2017)

[Original papers]

2014

1. Fujishima, T.; Suenaga, T.; Nozaki, T. (2014). Concise synthesis and characterization of novel *seco*-steroids bearing a spiro-oxetane instead of a metabolically labile C3-hydroxy group, *Tetrahedron Lett.*, **2014**, *55, 3805-3808.*
2. Fujishima, T.; Suenaga, T.; Nozaki, T. (2014). Synthetic strategy and biological activity of A-ring stereoisomers of 1,25-dihydroxyvitamin D_3 and C2-modified analogues, *Current Topics Med. Chem.*, **2014**, *14, 2446-2453.*
3. Liu, C.; Zhao, G.-D.; Mao, X.; Suenaga, T.; Fujishima, T.; Zhang, C.-M.; Liu, Z.-P. (2014). Synthesis and biological evaluation of 1α,25-dihydroxyvitamin D_3 analogues with aromatic side chains attached at C-17, *Eur. J. Med. Chem., **2014**, 85, 569-575.*

2013

4. Fujishima, T.; Nozaki, T.; Suenaga, T. (2013). Design and synthesis of novel 1,25-dihydroxyvitamin D_3 analogues having a spiro-oxetane fused at the C2 position in the A-ring", *Bioorg. Med. Chem.*, **2013**, *21*, 5209-5217.

[Review]

2016

5. Suenaga, T.; Nozaki, T.; Fujishima, T. (2016). Synthesis of novel vitamin D_3 analogues having a spiro-oxetane structure, *Vitamin*, **2016**, *90, 109-114.*

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Research

I. Gene expression analysis of mouse embryonic carcinoma P19 cells induced to form neural cells. (Takanobu Kobayashi and Hiroshi Miyazawa)

Mouse embryonic carcinoma P19 cells are pluripotent cells that can be induced to differentiate into multiple cell types by cellular aggregation in the presence of differentiating agents. When aggregated in the presence of all-*trans* retinoic acid (ATRA), P19 cells differentiate into neural cells (including neurons and glia cells), whereas the same cells aggregated in the presence of DMSO differentiate into cardiomyocytes. These cells can simulate the molecular and morphological events occurring during early embryonic development, and have been used extensively as a model to study the molecular mechanisms controlling the process of differentiation into cardiomyocytes or neural cells.

To identify the genes associated with induction of neural differentiation, we carried out a transcriptome analysis of P19 cells induced to form neural cells by ATRA. We employed the DNA microarray method, which can produce an accurate and derailed profile of gene expression. Numerous genes were activated in P19 cells in response to ATRA treatment. We compared the expression profiles from control (undifferentiated) and ATRA-treated P19 cells, which provided an abundance of information about the gene products involved in neural differentiation. We confirmed the sequential expression patterns of some genes over the course of differentiation. We are investigating the relationship between ATRA treatment and expression patterns of these genes, interactions with other factors, and functions in neural differentiation. These findings will provide useful clues to a more comprehensive understanding of the complex processes involved in the induction of neural differentiation. We are undertaking additional investigations to better understand the role played by these genes during the induction of neural differentiation.

Recently, we identified a candidate gene associated with induction of neural differentiation. We found that the expression of Csn3 was induced by all-trans retinoic acid (ATRA) during neural differentiation in P19 cells from our study using DNA microarray. We describe the induction mechanism of Csn3 transcription activation in this process. In conclusion, the Csn3 expression is upregulated via ATRA-bound RARα and binding of this receptor to the RARE in the Csn3 promoter region. This will certainly serve as a first step forward unraveling the mysteries of induction of Csn3 in the process of neural differentiation.

II. DNA Oxidation, Point Mutation and DNA repair. (Katsuhito Kino)

The genome is constantly assaulted by oxidation reactions that are likely to be associated with oxygen metabolism, and oxidative lesions are generated by many of these oxidants. Such genotoxin-induced alterations in the genomic message have been implicated in aging and in several pathophysiological processes, particularly those associated with cancer.

1. *Guanine Oxidation*

Photosensitized oxidation of guanine provides various oxidation products, including 8-oxoguanine (8-oxoG) and imidazolone. Riboflavin (vitamin B2) is known to be an effective photosensitizer for the oxidation of guanine. We have demonstrated: the user-friendly synthesis and photoreaction of a flavin-linked oligonucleotide; the practical synthesis of hydroxyethyl-flavin from commercially available riboflavin; and the preparation of a flavin-linked oligonucleotide using a phosphoramidite of hydroxyethyl-flavin. To demonstrate the usefulness of this method, the flavin-linked oligomer was synthesized. The flavin-linked oligomer and its complementary oligomer containing 8-oxoG were then irradiated under UV light (366 nm) at neutral pH. Enzymatic digestion of the irradiated mixture indicated that the 8-oxoG residue was oxidized to

imidazolone. These results demonstrated that 8-oxoG is effectively oxidized to imidazolone by photosensitization of the terminal flavin via a hole-transfer mechanism, and imidazolone is formed by one-electron oxidation of 8-oxoG at neutral pH.

In addition, 8-OxoG was specifically oxidized by iodine with aqueous KI. Under acidic conditions, the major product was dehydro-guanidinohydantoin. Under basic conditions, two diastereoisomers of spirohydantoin were chiefly obtained. In addition, unstable diimine was detected for the first time.

2. *Point Mutation by Guanine Oxidation.*

The guanine base (G) in genomic DNA is highly susceptible to oxidative stress because it has the lowest oxidation potential. Therefore, G-C-->T-A and G-C-->C-G transversion mutations frequently occur under oxidative conditions. One typical lesion of G is 8-oxoguanine (8-oxoG), which can pair with A, and this pairing may cause G-C-->T-A transversion mutations. Although the number of G-C-->C-G transversions is rather high under specific oxidation conditions such as riboflavin photosensitization, the molecular basis of G-C-->C-G transversions is not known.

We have shown that Iz is a key oxidation product of G when 8-oxoG in DNA photosensitized with riboflavin or anthraquinone. Primer extension experiments have demonstrated that Iz can specifically pair with G in vitro. Thus, specific Iz-G base pair formation can explain the G-C-->C-G transversion mutations that appear under oxidative conditions.

Moreover, we found that guanine is preferentially incorporated opposite 2,2,4-triamino-5(2H)-oxazolone (Oz) by eukaryotic DNA polymerases alpha, beta and epsilon, and we first propose the chemical structure of an Oz:G base pair having hydrogen bonds. Especially, since DNA polymerases alpha and epsilon play an important role in eukaryotic DNA replication, our results indicate that Oz is the premutagenic lesion that causes G:C-C:G transversions. Our results first clarify the mechanism of G:C-C:G transversions in eukaryote, and we mention the chemical consideration in guanine insertion opposite Oz. Thus we believe that our present study has novel insights into the molecular mechanism of point mutations underlying the first trigger which causes several diseases.

In addition to Oz, guanidinohydantoin (Gh)/iminoallantoin (Ia) and spiro-imino-dihydantoin (Sp) are known products of oxidative guanine damage. These damaged bases can base pair with guanine and cause G:C-C:G transversions. In this study, the stabilization energies of these bases paired with guanine were calculated in vacuo and in water. The calculated stabilization energies of the Ia:G base pairs were similar to that of the native C:G base pair, and both bases pairs have three hydrogen bonds. By contrast, the calculated stabilization energies of Gh:G, which form two hydrogen bonds, were lower than the Ia:G base pairs, suggesting that the stabilization energy depends on the number of hydrogen bonds. In addition, the Sp:G base pairs were less stable than the Ia:G base pairs. Furthermore, calculations showed that the Oz:G base pairs were less stable than the Ia:G, Gh:G and Sp:G base pairs, even though experimental results showed that incorporation of guanine opposite Oz is more efficient than that opposite Gh/Ia and Sp.

Next, we demonstrated that hNEIL1 and hNTH1 cleave Oz sites as efficiently as 5-hydroxyuracil sites. Thus, hNEIL1 and hNTH1 can repair Oz lesions. Furthermore, the nicking activities of these enzymes are largely independent of nucleobases opposite Oz; this finding indicates that removing Oz from Oz:G and Oz:A base pairs might cause an increase in the rate of point mutations in human cells.

3. *Product analysis of photooxidation in isolated quadruplex DNA*

(1) The formation of quadruplex structure changed the site reactivity and the kinds of guanine photooxidation products of d(TGGGGT). In quadruplex DNA, 8-oxo-7,8-dihydroguanine (8oxoG) and dehydroguanidinohydantoin (Ghox) were mainly formed, although 2,5-diamino-4H-imidazol-4-one (Iz) was mainly formed in single-stranded DNA. In addition, 3'-guanine was specifically oxidized in quadruplex DNA compared with single-stranded DNA, which depended on the localization of the HOMO.

(2) Guanine is the most easily oxidized among the four DNA bases, and some guanine-rich sequences can form quadruplex structures. In a previous study using 6-mer DNA d(TGGGGT), which is the shortest oligomer capable of forming quadruplex structures, we demonstrated that guanine oxidation products of quadruplex DNA differ from those of single-stranded DNA. Therefore, the photooxidation products of double-stranded DNA (dsDNA) may also differ from that of quadruplex or single-stranded DNA, with the difference likely explaining the influence of DNA structures on guanine oxidation pathways. In this study, the guanine oxidation products of the dsDNA d(TGGGGT)/d(ACCCCA) were analyzed using HPLC and electrospray ionization-mass spectrometry (ESI-MS). As a result, the oxidation products in this dsDNA were identified as 2,5-diamino-4H-imidazol-4-one (Iz), 8-oxo-7,8-dihydroguanine (8oxoG), dehydroguanidinohydantoin (Ghox), and guanidinohydantoin (Gh). The major oxidation products in dsDNA were consistent with a combination of each major oxidation product observed in single-stranded and quadruplex DNA. We previously reported that the kinds of the oxidation products in single-stranded or quadruplex DNA depend on the ease of deprotonation of the guanine radical cation (G•+) at the N1 proton. Similarly, this mechanism was also involved in dsDNA. Deprotonation in dsDNA is easier than in quadruplex DNA and more difficult in single-stranded DNA, which can explain the formation of the four oxidation products in dsDNA.

4. *Chemistry of flavins*

Photoirradiation in the presence of riboflavin led to guanine oxidation and the formation of imidazolone. Meanwhile, riboflavin itself was degraded by ultraviolet light A (UV-A) and visible light (VIS) radiation, and the end product was lumichrome. VIS radiation in the presence of riboflavin oxidized guanine similarly to UV-A radiation. Although UV-A radiation with lumichrome oxidized guanine, VIS radiation with lumichrome did not. Thus, UV-A radiation with riboflavin can oxidize guanine even if riboflavin is degraded to lumichrome. In contrast, following VIS radiation degradation of riboflavin to lumichrome, VIS radiation with riboflavin is hardly capable of oxidizing guanine. The consequences of riboflavin degradation and guanine photooxidation can be extended to flavin mononucleotide and flavin adenine dinucleotide. In addition, we report advanced synthesis; carboxymethylflavin was obtained by oxidation of formylmethylflavin with chlorite and hydrogen peroxide; lumichrome was obtained by heating of formylmethylflavin in 50% AcOH; lumiflavin was obtained by incubation of formylmethylflavin in 2 M NaOH, followed by isolation by step-by-step concentration.

III. Identification of novel low molecular compounds that inhibit binding of NF-□B to DNA (Takanobu Kobayashi)

The nuclear transcription factor-κB (NF-κB) is one of the central regulators of an organism's response to various stress signals. In response to an extracellular signal, NF-κB translocates into the nucleus, binds to DNA, and activates the transcription of specific genes. NF-κB regulates the transcription of a number of genes involved in immune and inflammatory pathways and in apoptosis. Dysregulation of NF-κB contributes to a variety of pathological conditions. Therefore, the down-modulators of NF-κB could have important therapeutic implications. One of the strategies for the down-regulation of NF-κB transcriptional activity is the specific inhibition of the DNA binding of NF-κB.

We have screened a virtual library using our structure-based computational screening method, thus enabling us to identify several compounds that inhibit DNA-NF-κB interactions. In our most recent studies, the inhibitory effects of the hit compounds selected from the virtual library were measured using fluorescence correlation spectroscopy (Olympus MF20) and an Electrophoresis Mobility Shift Assay. Using these methods, we found some compounds that inhibit the DNA-NF-κB interaction. We expect that these compounds may down-modulate the transcriptional function of NF-κB.

IV. Regulation of DNA replication machinery (Hiroshi Miyazawa)

DNA contains the genetic information which can be viewed as the organism's vital plan. Maintenance and replication of DNA, and the expression of the genetic information in DNA are the bases for life. In addition, information units of more than 10^9 are packaged and condensed in the nucleus of living cells. The condensation/decondensation of DNA molecules is dynamically repeated in growing cells during development and differentiation, necessitating strict control of the expression of genetic information.

Our purpose is to elucidate the functions of DNA replication factors and the proteins interacting with replication factors, and to ask how these factors act in various reactions occurring in DNA, such as DNA repair or transcription. We are investigating the behavior of these factors in nuclear structure, and studying how the DNA replication machinery is regulated during the cell cycle and cell differentiation.

So far, we have found that the second largest subunit of DNA polymerase ε (DPE2) interacts with SAP18, a polypeptide associated with the co-repressor protein Sin3. DNA polymerase ε is involved in chromosomal DNA replication, DNA repair and cell-cycle checkpoint control in eukaryotic cells. The Sin3 complex consists of several peptides containing the histone deacetylases, HDAC1 and HDAC2. By deacetylating histones in the chromosome, HDAC condenses chromatin structure, resulting in the repression of gene expression. The interaction of HDAC activity with replication factors predicts that DNA polymerase ε is involved in the maintenance of chromatin structure and transcriptional silencing during DNA replication. Thus DNA polymerase ε appears to be involved in epigenetic regulation. We are investigating how the interaction of DNA polymerase ε and the replication complex with proteins involved in epigenetic regulation (i.e. DNA methyltransferases, histone acetylases and deacetylases, and so on) change in the process of DNA replication and cell differentiation.

Publications (2012.4~2017.3)

[Original papers]

2017

1 Suzuki, M., Takeda, S., Teraoka-Nishitani, N., Yamagata, A., Tanaka, T., Sasaki, M., Yasuda, N., Oda, M., Okano, T., Yamahira, K., Nakamura, Y., Kobayashi, T., Kino, K., Miyazawa, H., Waalkes, MP., Takiguchi, M., " Cadmium-induced malignant transformation of rat liver cells: Potential key role and regulatory mechanism of altered apolipoprotein E expression in enhanced invasiveness." Toxicology, 2017, 382, 16-23

2 Kobayashi, T., "Expression and Regulation of *Tal2* during Neuronal Differentiation in P19 Cells" Yakugaku Zasshi, 2017, 137(1), 61-71

2016

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2 Suzuki, M., Kino, K.*, Kawada, T., Oyoshi, T., Morikawa, M., Kobayashi, T., Miyazawa, H. "Contiguous

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3 Kino, K.*, Sugasawa, K., Miyazawa, H., Hanaoka, F.* "2,2,4-Triamino-5(2H)-oxazolone is a Weak Substrate for Nucleotide Excision Repair." J. Pharm. Negat. Results, 2016, 7(1), 42-45

2015

1 Kobayashi, T., Suzuki, M., Morikawa, M., Kino, K., Tanuma, S., Miyazawa, H.* "Transcriptional Regulation of Tal2 Gene by All-trans Retinoic Acid (atRA) in P19 Cells." Biol. Pharm. Bull., 2015, 38, 248-256.

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7 Nakatsuma A, Wada S, Kamano J, Kiriyama Y, Kino K, Ninomiya M* The effects of herbal teas on drug permeability. Integr Mol Med, 2015, 3(1), 453-456.

8 Takahama K., Miyawaki A., Shitara, T., Mitsuya K., Morikawa, M., Hagihara M., Kino, K., Yamamoto A., Oyoshi T. G-quadruplex DNA- and RNA-Specific-Binding Proteins Engineered from the RGG Domain of TLS/FUS. ACS Chem. Biol., 2015, 10, 2564-2569.

9 Kino K.*, Suzuki M., Morikawa M., Kobayashi T., Iwai S., Miyazawa H. Chlorella virus pyrimidine dimer glycosylase and Escherichia coli endonucleases IV and V have incision activity on 2,2,4-triamino-5(2H)-oxazolone. Genes Environ., 2015, 37, 22.

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2 Morikawa M., Kino K.*, Senda T., Suzuki M., Kobayashi T., Miyazawa H. "Formation of a flavin-linked peptide." Molecules, 2014, 19(7), 9552-9561.

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2012

1 Kino K.*, Takao M., Miyazawa H., Hanaoka F.* (2012) "A DNA oligomer containing 2,2,4-triamino-5(2H)-oxazolone is incised by human NEIL1 and NTH1." Mutat. Res. 734 (1-2), 73-77.

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[Book/Review articles]

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3 Morikawa M., Kino K.*, Suzuki M., Kobayashi T., Komori R., Miyazawa H. (2012) "Oxidation of 8-oxoguanine with iodine and proposed mechanisms." Iodine: Characteristics, Sources and Health Implications, pp.121-133. (Nova Science Pub.)

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Research

Research Themes:

The mechanisms of immune cell trafficking and the regulation of immune responses are our main themes to clarify. Especially, we study roles of nuclear receptor ligands including vitamin A & D and various hormones in regulating immune functions especially in mucosal systems including the gut. By pursuing these biologically fundamental questions, we set a goal to establish a solid basis of new remedies and drug discovery for various diseases.

Recent Study:

For efficient immune responses, immune cells with proper functions need to migrate into right sites in the body. T cells, known as the control tower of the immune system, patrol the whole body along with the blood vessels and lymphatic vessels. However, they cannot migrate into non-lymphoid tissues before they are activated with antigen in the secondary lymphoid organs. Once they are activated and become effector or memory T cells, however, they can migrate into non-lymphoid tissues. They tend to migrate into the tissue that is associated with the secondary lymphoid organ where they are activated. This type of migration is called “homing”. For example, T cells that are activated with antigen in the small intestine-relate secondary lymphoid organs, Peyer’s patches (PP) and mesenteric lymph nodes (MLN), tend to migrate into small intestinal tissues including the lamina propria. In 2004, we found that vitamin A-derived retinoic acid is the physiological factor that imprints gut-homing specificity on T cells. We also found that subpopulations of dendritic cells (DC) in PP and MLN express the key enzyme of retinoic acid synthesis, RALDH (retinaldehyde dehydrogenase), and are capable of producing retinoic acid from vitamin A (retinol). They imprint T cells with the gut-homing specificity by delivering retinoic acid to T cells during antigen presentation. In 2006, we also found that a similar mechanism is involved in the imprinting of B cells with gut-homing specificity by a collaboration mainly with Dr. von Andrian’ s group and Dr. Adams’ group.

In 2009, we established a method for estimating the enzyme activity of RALDH in each DC, and identified the retinoic acid-producing subpopulation in MLN-DC and PP-DC. The RALDH2 isoform was mostly responsible for the activity. Depending on these results, we searched for the physiological factors that induce RALDH2 expression in DC in the gut or in MLN. We found that GM-CSF (granulocyte-macrophage colony-stimulating factor) plays a major role in the induction, and that retinoic acid itself plays a role as an essential cofactor. IL-4 and IL-13 exhibited effects similar to those of GM-CSF on RALDH2 expression, but are found to be dispensable by the analysis of their receptor-deficient mice. The stimulation through Toll-like receptors enhanced the RALDH2 expression in DC as well as DC maturation.

In 2007, several groups reported that the retinoic acid-producing DC also enhance the differentiation of $Foxp3^+$ inducible regulatory T cells (iTreg) and suppress that of pro-inflammatory Th17 cells. Accordingly, we found that GM-CSF-treated DC that expressed RALDH2 could enhance the differentiation of $Foxp3^+$ iTreg and suppress that of Th17. These results suggest that retinoic acid contributes to oral tolerance and regulation of immune responses to specific antigens. We have recently found that vitamin A deficiency affects not only the nature of T cells but also that of DC, and that MLN-DC in vitamin A deficient mice can induce oral antigen-specific $CD4^+$ T cells that produce high levels of IL-13 and tumor necrosis factor-α (TNF-α). Under vitamin A-deficient conditions, although it has been generally known that antibody responses are reduced, we found that markedly high levels of IgG1 antibody responses and IgE antibody responses against oral antigens can be induced. It is likely that these antibody responses involve the above-mentioned new IL-13-producing inflammatory helper T (Th) cells. Currently, we are investigating the molecular mechanism of differentiation of these Th cells and their role in allergic and inflammatory diseases.

We have been also analyzing some other aspects of retinoic acid effects on immune functions and regulation, including 1) The molecular mechanism of retinoic acid effects on the expression of gut-homing receptors on immune cells, 2) The role of a retinoic acid-metabolizing system in the regulation of T cell functions, 3) Amplification and disruption of retinoic acid signals by RXR ligands and environmental chemicals, 4) The molecular mechanism of RALDH isoform 2 (RALDH2, encoded by *Aldh1a2*) expression

in DC and the roles of a retinoic acid-bound RAR/RXR heterodimer and a retinoic acid response element (RARE) half-site at the proximal promoter of the *Aldh1a2* gene. The RARE half-site in this gene promoter was commonly found in many animal species.

We found that, in vitamin A-deficient mice, dextran sulfate sodium (DSS) induced more severe colitis, and a higher rate of development of colorectal carcinoma with colitis following treatment with azoxymethane, compared with vitamin A-supplemented mice. Therefore, vitamin A is likely to inhibit the development of chronic inflammation and cancer. These vitamin A effects may also be dependent on retinoic acid production. As we mentioned above, GM-CSF and retinoic acid itself play important roles in the development of retinoic acid-producing DC, and Toll-like receptor-mediated stimulation enhances their maturation and retinoic acid-producing capacity. However, we recently found that Toll-like receptor stimulation also induced production of inflammatory cytokines. As retinoic acid-producing RALDH2^{high} MLN-DC in steady-state mice do not produce inflammatory cytokines, we searched for a stimulation condition that can induce maturation in GM-CSF/retinoic acid-treated semi-mature DC and can enhance their RALDH2 expression without inducing inflammatory cytokines. We found that stimulation with immobilized proteins such as E-cadherin/IgG-Fc chimeric protein fulfilled the requirement. These proteins induced signals through integrin β1 in DC. E-cadherin expressed on epithelial cells in gut tissues might contribute to the development of the RALDH2^{high} DC. These DC induced in vitro could efficiently induced gut-homing iTreg, and could significantly suppress DSS-induced colitis.

Publications

＊ 2012-2016

2016

1. Yokota-Nakatsuma, A., Ohoka, Y., Takeuchi, H., Song, S.-Y., and Iwata, M.: Beta 1-integrin ligation and TLR ligation enhance GM-CSF-induced ALDH1A2 expression in dendritic cells, but differentially regulate their anti-inflammatory properties. ***Sci Rep.*** 6:37914 (2016).
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Research

Research project: Analysis of the molecular mechanism by which extracellular acidification induces inflammatory responses in rheumatoid arthritis synovial cells. (Nochi)

The local acidification of extracellular pH is caused by augmentation of cell proliferation as observed in the cancer tissue and the inflammatory site. In RA, the proliferation of synovial cell is abnormally augmented and the pH of synovial fluid from RA patient is lower than that of normal synovial fluid. Therefore, we examined the possibility that the intraarticular acidification may affect the inflammatory responses and contributes to exacerbation of pathological condition in RA. We found that extracellular acidic pH induced ADAMTS-4 expression through Gq-coupled proton-sensing receptor (OGR1) in RA synovial cells. Furthermore, extracellular acidic pH-induced and ADAMTS-4 expression were markedly inhibited by siRNA targeted for the OGR1 receptor and the inhibitors for $G_{q/11}$ protein and p38 MAPK. We conclude that the OGR1/ $G_{q/11}$/p38 MAPK or NFκB pathway regulates ADAMTS-4 expression under acidic condition. To examine details of the intracellular signaling mechanism by which ADAMTS-4 induction are regulated under acidic circumstances, further studies are now under way.

Research project: characterization of retinoid X receptor signaling on T cell differentiation. (Takeuchi)

Retinoic acid (RA) is an immune-modulating molecule, and its signaling is known to affect T cell differentiation. It can enhance differentiation toward regulatory T cell (Treg), and suppress that toward Th17. RA receptor is consist of two components, RAR and RXR. Although RXR has a ligand-binding domain, it does not bind RA at physiological condition. It is not well-studied whether RXR-specific signaling can affect T cell differentiation.

We found that RXR signaling actually played some roles on T cell differentiation. RXR signaling dramatically enhanced RA-mediated Treg differentiation. On the other hand, it suppressed that of Th17 in cooperation with some nuclear receptor signaling. The effect of RXR signal was observed in vivo as well as in vitro. Thus, this finding can apply to develop new methods to regulate immune-response and inflammatory diseases.

Research project: Analysis of the molecular mechanism of GABARAPs in autophagy. (Kiriyama, Nochi)

Astrocytes play several important roles in the central nervous system: providing nutrients to neurons, forming the blood-brain barrier, maintaining the extracellular ion balance, surrounding and maintaining synapses, and participating with neurons in the processing of information. Thus, optimal function of the brain depends on maintainance of homeostasis of astrocytes as well as neurons from stresses. Cell survival and maintainance of homeostasis depends on removal of damaged cellular cytosolic components. Macroautophagy (hereafter referred to as autophagy) is the process by which defective proteins and damaged organelles are isolated and degraded to generate new proteins and organelles under a variety of stresses. Moreover, impairment of autophagic processes results in neurodegeneration

We found that reactive oxygen species (ROS) and the mitochondrial damage leads to the induction of autophagic flux and the upregulation of gamma-aminobutyric-acid-type-A receptor-associated protein (GBARAP) subfamily proteins in C6 glioma cells. GABARAPL1 mRNA among GABARAP subfamily members were highly upregulated by ROS. GABARAPL1 are associated with both autophagosome formation and carrying target proteins to autophagosomes. Thus, the expression of the GABARAP subfamily members may depend on discrete regulations of the expression of mRNA and protein, and the GABARAP subfamily members may have discrete functions in autophagy. Further studies on the mechanism of GABARAP subfamily members in autophagy are under way.

Publications (2012~2017)

[Original papers]

2016

1. Yokota-Nakatsuma, A, Ohoka, Y., Takeuchi, H., Song. S.-Y.

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1. Takeuchi, H., Yokota-Nakatsuma, Y., Ohoka, Y., Kagechika, H., Kato, C., Song, S.-Y., and Iwata, M.: Retinoid X receptor agonists modulate Foxp3^{+} regulatory T cell and Th17 cell differentiation with differential dependence on retinoic acid receptor activation. ***J Immunol*** 191(7): 3725-3733 (2013).

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Seeing through the brain mechanism for memory

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Research

Main Project from 2016 (Maki K. Yamada, Shuntaro Kohnomi)

Seeing through the brain mechanism for memory using transgenic mice

Background

We showed that an F-actin stabilizing protein CapZ accumulated and stayed around synapses (spines) that underwent long-term potentiation (LTP) (Genes to Cells 2010), thus the place for memory-related change in neuronal synapses is expected to be labeled by EGFP-CapZ even in-vivo. On the other hand, we published another paper (Cerebral cortex 2009) suggesting that memory-coding neurons are included in a part of Arc-expressing neurons, thus, Yamada made the transgenic mouse of EGFP-CapZ driven by the Arc-promotor, Arc::EGFP-CapZ, to mark the memory coding synapses and neurons. In the resulting mouse having blight fluorescence and normal learning ability, the EGFP-fluorescence is mainly found in some of synaptic structure, spines, and the cell body.

Till now,

1. In-vitro confirmation; It was confirmed that the LTP-inducing stimuli increased the level of the green fluorescence in a part of the synaptic structure (spines) and the neuronal cell body in the hippocampus sections from the TG having Arc::EGFP-CapZ (ACE-TG) as expected.

2. In-vivo observation; we observed that a small number of strongly green neurons emerged after the visually-cued learning in the primary visual cortex of the ACE-TG.

3. Ex-vivo analyses; we analyzed the changes of the green neurons and spines in the primary visual cortex by making fixed brain sections from ACE-TG with or without the same visual learning and then found the differences.

Future plans: Seeing through the brain mechanism for memory by multidisciplinary approach.

References from Yamada's group

1.Cereb.Cortex

Experience-dependent, rapid structural changes in hippocampal pyramidal cell spines.

Kitanishi T, Ikegaya Y, Matsuki N, Yamada MK*

Cerebral cortex (New York, N.Y.) 19(11) 2572-2578 (2009)

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2.Genes Cells

Activity-dependent localization in spines of the F-actin capping protein CapZ screened in a rat model of dementia.

Kitanishi T, Sakai J, Kojima S, Saitoh Y, Inokuchi K, Fukaya M, Watanabe M, Matsuki N, Yamada MK* Genes to cells : devoted to molecular & cellular mechanisms 15(7) 737-747 (2010)

http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2443.2010.01411.x/pdf

Kohnomi's project with students

The stress causes most of psychiatric diseases. Aiming to clarify their etiology, we used the chronic restraint stress model. We examined the influences of this stress on the modulation by “monoamines” in the medial prefrontal cortex or nucleus accumbens of the stressed mice.

Kuriu's project with students

Primary cultures of neurons dissociated from the brain of wild type and AD model mice were analyzed using confocal imaging and electrophysiology, which allow us to study the formation of excitatory and inhibitory synapses.

Publications (2012～2017)

[Original papers]

2016

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2. Yamada, M. K.* (2016) A link between vascular damage and cognitive deficits after whole-brain radiation therapy for cancer: A clue to other types of dementia? Drug Discov. Ther., Vol.10. p 74-78
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Research

Research Themes:

It's clear that the future direction of the Pharmaceutical science is "the development of therapeutic method of human diseases based on the understanding of pathophysiology at molecular level". This direction is also one of the most important bases of Pharmaceutical education. Thus we set final goal of our research on the integrative understanding of the pathogenesis of human diseases from molecular to individual level. To pursue this task, animal models for human diseases are beneficial experimental tools. From the analyses of these animals, we have a chance to combine analyses of molecular and cellular level with clinical changes at individual level. Further, these animals are valuable, because we can obtain most early changes in the pathogenesis of diseases, which are hardly examined in human patients. Thus one of the main methods of our division is histopathological analysis of these animals. These analyses require systemic approaches from macroscopic anatomy, conventional histological methods, immuno histochemical and *in situ* hybridization histochemical methods using light microscopy and electron microscopy. Now we are trying to establish a system for analyses integrating these lesions identified by histological techniques with biochemical and molecular biological analyses. We also pursue cooperative research projects with medical institutions outside our university for analyses using human materials that fulfill ethical criteria, depending on the progress in each research project. These trials using good animal models for human disease are expected to contribute to the better understanding of the pathophysiology of human disease. Following are the detailed information of each research project.

Effects of modified gene expression of lipid metabolism-related enzymes on remyelination following cuprizone-induced demyelination.

Pathogenesis of demyelinating disease such as multiple sclerosis is not yet clear and effective therapy is not established. Lipid is a major component of myelin and remyelination involves various kinds of lipid metabolism-related enzymes, which can be therapeutic targets of demyelinating disease. Our previous study revealed that the expression of lanosterol 14-α Demethylase (LDM, CYP51), stearoyl-CoA desaturase (SCD), lipidosin is up-regulated during the process of remyelination, suggesting that activation of these lipid metabolism-related enzymes can accelerate remyelination. To further pursue functional roles of these enzymes in remyelination, following studies were done.

1. Effects of up-regulation of LDM on remyelination

LDM is the only cytochrome P450 enzyme that is involved in cholesterol biosynthesis in eukaryotes. It is expressed abundantly in the liver and moderately in the brain. Cholesterol is not only a major component of plasma or endoplasmic reticulum membrane but also an essential component of myelin sheath in the central and peripheral nervous system. Cholesterol required for myelination in the brain is newly synthesized in oligodendrocytes, which form myelin sheaths, because cholesterol synthetized in the liver can't cross the blood brain barrier. Previously, our immunohistochemical and biochemical analyses indicated that LDM was predominantly expressed in oligodendrocyte and Schwann cell in the central and peripheral nervous system, respectively, and that its expression increased in the process of myelination during postnatal development and remyelination in the experimental demyelination-remyelination, which was induced by feeding ICR mice with a diet including cuprizone, then with a normal diet. These results suggest that augmentation of LDM expression in oligodendrocyte may have therapeutic significance in demyelinating disease.

In order to reveal whether LDM is critical enzyme for remyelination, we generated LDM transgenic mouse (LDM-Tg) driven by myelin proteolipid protein (PLP) promoter, which is oligodendrocyte-specific and PLP shows a similar expression pattern of LDM in the brain during myelination and remyelination. The oligodendrocyte-specific LDM expression cassette was constructed from PLP promoter with a length of 10 kbp, which was cloned from the first intron of mouse genomic DNA, LDM cDNA, which was cloned from total RNA derived from mouse brain, and polyadenylation signal sequence derived from SV40 late polyadenylation signal. The cell specificity of this expression cassette was confirmed by comparing the effect in culture cells,

positive expression in Oli-neu cells derived from mouse oligodendrocyte and negative expression in HEK293T. This construct DNA was injected into pronuclei of fertilized egg and then LDM-Tg was obtained, which shows oligodendrocyte-specific overexpression of LDM with genetic background of C57BL/6.

Using this LDM-Tg, following data were obtained.

(1) Western blot analyses of isolated proteins from brain, spinal cord, liver, kidney, lung, testis, adrenal gland, thymus, spleen, small intestine, gastrocnemius muscle, heart of LDM-Tg and wild type mouse (WT, C57BL/6) revealed the expression of LDM in all the organs except lung and small intestine, but increased expression of LDM was confirmed only in the brain and spinal cord.

(2) Western blot analyses using anti-PLP antibody revealed the expression of PLP only in the brain and spinal cord. Double immunohistochemcal analyses using anti-LDM antibody and marker antibodies specific for oligodendroglia showed that stronger LDM-immunoreactivity was localized in the cytosol of oligodendroglia of LDM-Tg. These data strongly suggest that established LDM-Tg shows PLP-promoter dependent, oligodendroglia specific overexpression of LDM in the brain and spinal cord.

(3) LDM-Tg and WT showed no difference in myelination during postnatal development, indicating that overexpression of LDM unlikely induce hypermyelination.

(4) After 6-8 weeks' feeding a diet containing cuprizone, demyelination in the corpus callosum was moderate in LDM-Tg, while it was severe in WT. These data suggest that reactive remyelination that occurs during the progress of demyelination is accelerated in LDM-Tg as compared with WT, probably due to accelerated cholesterol biosynthesis. Moreover, difference in the degree of remyelination between LDM-Tg and WT was prominent at one week after changing to normal diet, but not so prominent at four weeks. These data suggest that early stage of remyelination induced by feeding a normal diet was accelerated in LDM-Tg mice as compared with WT mice.

(5) Taken together, LDM may be one of critical enzymes for remyelination and a promising target of drug-development for the therapy of demyelinating disease.

2. Effects of targeted disruption of lipidosin gene on remyelination.

Lipidosin is an enzyme with long-chain acyl-CoA synthetase activity and expressed only in the brain, adrenal gland and testis, all of which are impaired in adrenoleucodystrophy. Using lipidosin KO mice, following results were obtained.

(1) Lipidosin KO mice and WT mice showed no difference in myelination during postnatal development.

(2) Remyelination was significantly delayed in lipidosin KO mice as compared with WT mice, while the degree of demyelination was comparable between both mice.

These data suggest that modified gene expression of LDM and lipidosin can affect remyelination, though such modification does not affect normal myelination and that enhancement of these enzymes can have therapeutic effects for demyelinating disease.

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2016

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2. Yokota-Nakatsuma A, Ohoka Y, Takeuchi H, Song S-Y, and Iwata M. Beta 1-integrin ligation and TLR ligation enhance GM-CSF–induced ALDH1A2 expression in dendritic cells, but differentially regulate their anti-inflammatory properties. Sci Rep 6:37914. doi: 10.1038/srep37914 (2016)

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Research

【Research aims】

Our research goal is the novel molecular target for new antiepileptic drugs. To achieve this goal, we are working on molecular mechanism for the epileptogenesis of partial and generalize epilepsy through the several approaches such as pharmacological, behavioral, cell biological, biochemical and *in vivo* imaging techniques.

【Research Scopes】

1. Prevention of status epilepticus-induced brain edema and neuronal cell loss by new antiepileptic drugs.

Status epilepticus (SE) refers to neurologic emergencies that may lead to death or permanent neurologic injury. To avoid life threating injury, patients must be properly and quickly treated. Furthermore, SE causes 3~5% of symptomatic epilepsy (~35% of epileptic syndromes), thus SE patients are at a high risk of developing acquired epilepsy (Hesdorffer, 1998; Temkin, 2003; Jacobs et al., 2009). The management of SE is important to prevent mortality and the development of post-SE symptomatic epilepsy. Seizures must be treated as soon as possible and benzodiazepines (lorazepam or diazepam) are typically administered as first-line antiepileptic drugs (AEDs). However, when these drugs fail, second-line AEDs (phenytoin; PHT, fosphenytoin; fosPHT, valproate; VPA, and midazolam) are administered in refractory SE prior to giving phenobarbital; PB (Manno, 2011). Various clinical trials have indicated that conventional AEDs (e.g., DZP, PB, VPA, or PHT) suppressed acute seizures, but thus far there has been no success at preventing the development of post-SE acquired epilepsy under various conditions (Temkin, 2001; 2003; 2009). Although the mechanisms underlying the development of acquired epilepsy as part of the epileptogenic process are not well understood, the lack of efficacy of the AEDs suggests that the biological mechanisms of the acquired epilepsy process may be quite different from that of the established epileptic brain (Pitkanen et al., 2009).

Levetiracetam ([(S)-α-ethyl-2-oxo-1-pyrrolidine acetamide]) with broad-spectrum antiepileptic effects is an established second-generation AED that is widely used in patients with either generalized or partial epilepsy (Lyseng-Williamson, 2011). In addition, levetiracetam is one of currently available candidates as second-line AED for SE (Manno, 2011) and as an anti-epileptogenic drug (Pearl et al., 2013; Klein, et al., 2012). Animal studies have shown that levetiracetam possesses anticonvulsant activity and results in neuroprotective effects (Mazarati et al., 2004; Zheng et al., 2010). In addition, levetiracetam has been considered for the treatment of pilocarpine (PILO)-SE due to its anti-epileptogenic effects in basic and clinical studies. Two phase II clinical trials for levetiracetam indicated the possibility that it may decrease the risk of acquired epilepsy or prevent the development of acquired epilepsy (Pearl et al., 2013; Klein, et al., 2012). However, the previous evidence in SE animal models has been conflicting and whether levetiracetam can prevent or modify epileptogenesis remains controversial (Löscher et al., 1998; Glien 2002; Klitgaard, Pitkanen, 2003; Stratton et al., 2003; Gibbs et al., 2006; Brandt, et al., 2007).

Temporal lobe epilepsy (TLE) is the most frequent type (75%) of symptomatic partial epilepsies that originate from the limbic regions (e.g., hippocampus and amygdala) after an initial brain insult, such as SE, stroke, and traumatic brain injury (TBI). Additionally, it is also one of the most refractory forms of epilepsy with approximately 30% of patients being unresponsive to AEDs (Engel, 1996; Kwan and Brodie, 2000). In this present study, we used PILO-induced SE mice as a model of TLE to determine the effects of repeated administration of high-dose levetiracetam after the termination of SE by DZP. We observed that repeated

high-dose levetiracetam prevented the development of brain edema in the limbic regions at the initial period of post-SE, and the incidence of spontaneous recurrent seizures. In the present study, we determined the possible molecular and cellular mechanisms of LEV treatment after termination of SE. To assess the effect of LEV against the brain alterations after SE, we focused on blood-brain barrier (BBB) dysfunction associated with angiogenesis and brain inflammation. The consecutive treatment of LEV inhibited the temporarily increased BBB leakage in the hippocampus two days after SE. At the same time point, the LEV treatment significantly inhibited the increase in the number of CD31-positive endothelial immature cells and in the expression of angiogenic factors. These findings suggested that the increase in neovascularization led to an increase in BBB permeability by SE-induced BBB failure, and these brain alterations were prevented by LEV treatment. Furthermore, in the acute phase of the latent period, pro-inflammatory responses for epileptogenic targets in microglia and astrocytes of the hippocampus activated, and these upregulations of pro-inflammatory-related molecules were inhibited by LEV treatment. These findings suggest that LEV is likely involved in neuroprotection via anti-angiogenesis and anti-inflammatory activities against BBB dysfunction in the acute phase of epileptogenesis after SE.

2. Study on the relationship between blood brain barrier (BBB) disruption and epilepsy.

In recent years, it has been well recognized that the therapies for epilepsy by the AEDs, which is represented by valproate, have been definitely effective. On the other hand, no less than 30% of epileptic patients were intractable, so there are difficulties in achievement of high level of QOL for them. In order to dissolve this problem, the development of new AEDs with novel mechanisms is an important for drug-resistant patients. We aim to find out the novel molecular target for new drugs. Recently, we have focused the relationship between BBB disruption and generalized epilepsy. Although conventional evaluation methods of BBB disruption are to measure the diffusion of low molecular weight dye (ex. Evans blue) to brain parenchyma, they are not available in animals alive. In our laboratory, the spatial and sequential changes of the BBB disruption in PTZ-induced convulsive alive mice were elucidated by the technique of gadolinium-enhanced magnetic resonance imaging using the MRI for rodent (MRminiSR, 1.5T). In addition, we have investigated the involvement of NO in the BBB disruption in generalized epilepsy.

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Research

1. Integrated study on pharmacokinetics and pharmacodynamics for efficient drug discovery and on the optimum drug therapy
2. Basic and clinical analysis of drug-metabolizing enzyme and transporters influencing pharmacokinetics and pharmaco-dynamics
3. Basic research on the toxicity mechanism of drugs, xenobiotics and their active metabolites
4. Basic research for synthesis of COA-Cl (2-Cl-C.OXT-A) analogs, and their tube formation activities of human umbilical vein endothelial cells (HUVEC)
5. Design, synthesis, and anti-HBV activity of heterocyclic derivatives
6. The development of novel reaction for the heterocyclic bioactive substances
7. Curcumin and doxorubicin co-encapsulation liposome effects on cell growth, apoptosis, and angiogenesis

The elucidation of relationship between pharmacokinetics (PK) and pharmacodynamics (PD) of drugs is critical not only for the discovery of novel drugs but also for their optimum uses in clinical settings. The effects of clinically used drugs are influenced by drug-metabolizing enzymes and drug-transporters. Therefore, one of our major research projects is to push an integrated analysis of PK and PD for investigational drugs by characterizing *in vivo* drug-metabolizing enzymes and drug-transporters under physiological and pathological conditions.

We are also conducting the research of mechanism(s) for the decrease in levels of serum thyroid hormones by xenobiotics and their active metabolites, and the species difference and extrapolation to human in these compounds-induced alteration of the hormone levels.

In addition to these projects, we have also focused on simultaneous determination of polybrominated diphenyl ethers (PBDEs), hydroxylated (OH-) and methoxylated (MeO-) PBDEs found in marine sponge by atmosphere pressure chemical ionization tandem mass spectrometry (APCI-LC/MS/MS), and immunotargeting of liposome to tumor and endothelial cell expressed membrane type-1 matrix metalloproteinase (MT1-MMP).

Stimulator of angiogenesis: Stimulators of angiogenesis are sometimes desired for clinical treatment of diseases evoked by an impaired blood supply including ulcers associated with diabetes or burn wounds. However, availability of stimulators is few till date because of their size. Most of the stimulators known are endogenous large molecules like VEGF and FGF. Those are expensive proteins, hard to synthesize, and not so stable. We developed 2-Cl-C.OXT-A as a stable candidate compound. This compound strongly stimulates the tube formation of HUVEC. Its maximun potency at 100μM was stronger than VEGF (10ng/mL). 2-Cl-C.OXT-A will be applicable to medicine instead of endogenous growth factors such as VEGF and/or FGF.

Effect of uracil analog against HIV-1: Human immunodeficiency virus type 1 (HIV-1) contains an important enzyme, reverse transcriptase (RT), which catalyzes the conversion of the viral genome RNA into the double-stranding DNA. Since this process is essential for viral replication, many drugs targeting this enzyme have been developed. Within the class of the anti-HIV agents which inhibit reverse transcriptase, non-nucleoside reverse transcriptase inhibitors (NNRTIs) are rapidly increasing. It is interesting that some NNTRIs have an aromatic group at the 6 position of uracil. Under the background of these reports, we undertook a search for an anti-HIV agent by the SAR of the 1,3-disubstituted uracil. Two compounds showed excellent anti-HIV-1 activity with moderate cytotoxicity.

Publications (2012-2017)

[Original papers]

2017

1. Sakakibara, N., Igarashi, J., Takata, M., Konishi, R., Kato, Y., Tsukamoto, I. (2017). Synthesis and evaluation of novel cyclopropane nucleoside as potential tube formation agents. Chem. Pharm. Bull., 65,504-510.

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2012

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Research

We have the research philosophy for Laboratory of Pharmaceutics, Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University. Research projects were chosen based on the philosophy. The selected projects and those results were as follows:

(1) Development of a novel dosage form containing fluticasone propionate for inflammatory bowel diseases

Crohn's disease and ulcerative colitis are the two primary types of inflammatory bowel diseases (IBD). Glucocorticosteroids were the standard treatment for IBD, but due to adverse events, their use was limited. However, new formulations of glucocorticosteroids have been developed to reduce systemic action. Fluticasone propionate (FLT) is an inhaled corticosteroid with high anti-inflammatory potency, used for the topical treatment of asthma. The purpose of this project was the design and preparation of a new FLT dosage form for topical treatment of IBD. The physicochemical property of FLT was very important for the dosage form design. The information for FLT was not enough, so we started the determination of the property. Chemical structures for degradation products of FLT in an alkaline solution were found by the support of Laboratory of Pharmacognosy and Natural Products Chemistry, which results were submitted and accepted. Basic studies for developing the new formulation were maintained.

(2) Effect of the simple suspension method on the dissolution behavior

The simple suspension method is usually used. However, there was no report regarding the change of the dissolution property of the drug. The purpose of this project was a comparison of the dissolution profile between original and generic drugs after applied the simple suspension method. We evaluated the pharmaceutical preparations with candesartan cilexetile and valsartan.

(3) Cleaning validation for machines used in the dispensary of pharmacy

When machines used in the dispensary, for an example, a dividing and packing machine was applied for a granule or a powder, it was easily considered that the little amount of the granule or powder was left in the machine. Therefore, cleaning the machine was required. This cleaning will be performed according the procedure which is decided by each pharmacy. In the case of a pharmaceutical plant, cleaning validation was required for machines for manufacturing pharmaceutical preparations by GMP. The purpose of this project is to introduce the concept of cleaning validation to pharmacies. The preparations indicating the higher residual percent were researched on the basis of the result of questionnaire for pharmacists, which were the preparations of pranlukast hydrate, ketotifen fumarate, acetaminophen, and nicotinamide. The residual amount of the drugs in the machine were determined.

(4) Degradation rate of ebastine in an acidic solution and the effect of cyclodextrins (CDs) on the its degradation rate

Degradation rate of ebastine in an acidic solution and the effect of cyclodextrins on its degradation rat were examined. In addition, the degradation rates of CDs were determined. A paper was submitted and accepted.

(5) Development of novel pharmaceutical technology for poorly water-soluble drugs

To enhance oral bioavailabilitiy of poorly water-soluble drugs such as curcumin etc, we tried to prepare powders or water-based suspensions contain novel nano-particles by build-up or break-down methods, and estimated its particle characteriscics.

(6) Evaluation of powder preparations with sildenafil citrate for newborns in NICU

Sildenafil is a phosphodiesterase type 5 inhibitor that selectively reduces pulmonary vascular resistance in animal models and adult humans. Recent studies reported that the administration of sildenafil significantly increased oxygenation and reduced mortality with no clinically important side effects in infants with persistent pulmonary hypertension of the newborn.

A pharmaceutical preparation containing sildenafil citrate (SIL) for pulmonary arterial hypertension, Revatio Tablets 20 mg from Pfizer Japan Inc., is available for adults in Japan, whereas that for children is not. Therefore, when sildenafil is administered to infants with persistent pulmonary hypertension of the newborn, a Revatio Tablets 20 mg is ground in a mortar to make a powder. Lactose is added to the powder as a diluent, and is mixed well in the mortar. The mixed powder is packaged for each dose using an automatic packaging machine. The contents of the drug in the

packages were determined by HPLC. A part of this data was submitted and published. From this study the decrease of the content of sildenafil in the powder. A method for preventing the decrease was developed and reported.

(7) Improvement of jellies with alendronate sodium

A jelly preparation of alendronate sodium is bitter, so the aim of this study is the improvement of the jelly. A jelly preparation with ethyl cellulose film on the inside jelly was prepared. The dissolution profile from the jelly was determined.

Publications (2012~2017)

[Original papers]

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Research

(1) Patient–Healthcare Professional Relationship

Patients accept or refuse their medication therapy based on their personal beliefs. We analyze relationship between patient's perceptions of medication therapy and their behavior such as medication adherence.

We developed Medication Acceptance, Preference and Adherence Scale (MAPAS), which assessed each patient's beliefs, values and ideas concerning their acceptance and preference for medications and treatments. We found that patients' dissatisfaction consistent determinants of intentional non-adherence to medication, but not unintentional non-adherence. In addition, we found that cancer patients prefer aggressive therapies, even when self-estimations of ADR endurance are not very high, especially if they have been receiving chemotherapy for a short period of time.

(2) Pharmacoepidemiology Study Encouraging Proper Use of Medication

Post-marketing surveillance is important because it can evaluate medication use in the real world.

We surveyed use of medication with driving with prohibitions or cautions in outpatient settings for patients aged 25 years and older using the National Health Insurance Claims Database. Of outpatients aged 25 years and older who were administered medications, 73% outpatients were given the medications with cautions or prohibitions on driving. For the elderly, prescriptions were found with dosages that often exceeded the recommended limits.

(3) Bio- and Chem-Informatics and Computational Sciences of Traditional Medication Systems and Medicinal Resources

We perform the informatics and computational sciences based on the evidences related to medical and pharmaceutical issues. This research has been demonstrated by biological and chemical experiments, database construction, and factor analysis by multivariate statistical analysis. In recent studies, we have focused on the theoretical analysis of the traditional and empirical medication system in Kampo (traditional Japanese medicine), and the transcriptome and metabolome analyses of medicinal bioresources and a model organism.

(4)Modulation of multi-drug resistance related protein transport by interaction with dietary supplements

An interaction is taken to be the situation in which administration of a drug or substance induces changes in the pharmacokinetics of another simultaneously administered drug – by increasing either the plasma or intracellular concentration of the latter, and thus giving rise to the possibility of an adverse reaction.

The ABC-transporter superfamily, which functions as a drug efflux pump, is known to limit the absorption of a variety of drugs. We investigated the effects of food extracts on anticancer drug transport by the multi-drug resistance related proteins (MRPs). MRPs are efflux transporters expressed in human glioblastoma cell line T98G. The effects on MRP mediated transport were also evaluated using calcein, which is the substrate of MRP. Acute exposure to kaempferol caused a concentration-dependent decrease in the extracellular efflux of calcein compared with the control As for the simultaneous exposure to kaempferol and cisplatin, the cytotoxicity of cisplatin was expected to be potent because MRP and glutathione *S*-transferases (GST) are both inhibited by kaempferol. However, the cytotoxicity of cisplatin decreased

Western blot analysis and reverse transcription–polymerase chain reaction (RT–PCR) showed that treatment with 10 and 20 μM kaempferol for up to 72 hr was able to significantly lower MRP2 expression, whereas increased expression in a concentration-dependent on GST mRNA and protein levels. Furhermore, GST was strongly activated in T98G cell treated with

kaempferol.

The results of the study also point to possible kaempferol-drug interaction, especially when the cytotoxicity of anticancer drugs are dependent on glutathione *S*-transferases and MRP-mediated transport processes. Hereafter, these possible efficacies need to be examined under in vivo conditions in detail.

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＊ 2012-2016

[Original papers]

2016

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3. Sadamoto, H., Takahashi, H., Okada, T., Kenmoku, H., Toyota, M., and Asakawa, Y. (2012). *De Novo* sequencing and transcriptome analysis of the central nervous system of mollusc *Lymnaea stagnalis* by deep RNA sequencing. PLoS One 7(8), e42546.

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3. Iihara, N., and Kirino, Y. (2014). “A community electronic prescription system connecting physicians, pharmacists, and patients, and utilization of clinical information” YAKUGAKU ZASSHI 134(5), 589-593.

[Books]

1. Okada, T., and Noji, M. (2014). Metabolome analysis of medicinal plants and crude drugs –a study case of *Ephedra* plants by comprehensive metabolite analysis using mass spectrometry and multivariate statistical analysis–. (Chapter 1 in Part III) In: Kawahara, N. (Sv.) Recent progress of studies on medicinal plants and crude drugs –cultivation and quality evaluation of medicinal plants, and development of Kampo products– (in Japanese). CMC Publishing, Tokyo, pp. 122–131.
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Research

Theme 1. The molecular mechanism of complexin (Hiroshi Tokumaru, Hisayo Sadamoto)

Action potential-evoked neurotransmitter release is triggered by Ca^{2+} influx through voltage-gated calcium channels located next to the active zone. The increase in Ca^{2+} concentration initiates rapid signaling cascades that lead to the exocytosis of synaptic vesicles containing high concentrations of neurotransmitter. Two soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins from the presynaptic membrane, syntaxin 1 and SNAP-25, and one SNARE protein from the synaptic vesicle membrane, synaptobrevin-2 (also known as VAMP-2), form a four-helix bundle (called the *trans*-SNARE complex or SNAREpin) that catalyzes membrane fusion. The synaptic vesicle protein synaptotagmin 1 (Syt1) serves as a major Ca^{2+} sensor for fast action potential-evoked synaptic vesicle exocytosis. The rapid interactions between Syt1, the SNARE complex and membrane phospholipids induced by Ca^{2+} are critical for membrane fusion.

The precise control of evoked neurotransmitter release requires several cytosolic proteins including complexin (also known as synaphin). Complexin and its binding to the SNARE complex are critical for fast neurotransmitter release, as demonstrated by studies in knockout mice and in *Drosophila* mutants. In addition, intra-presynaptic terminal injection of a SNARE-binding domain peptide that blocks complexin binding to the SNARE complex also inhibits rapid neurotransmitter release. Despite this evidence, complexin's function remains controversial. Furthermore, recent studies suggest that complexin contains several functional domains that either stimulate or inhibit neurotransmitter release. Thus, the function of complexin is likely more complex than expected from its small size (134 aa for mammalian complexin 1 and 2).

Like Syt1-deficient mice, complexin 1/2 double-knockouts exhibit an impairment of action potential-evoked synchronous (but not asynchronous) neurotransmitter release. However, an important difference exists between the two types of knockout mice: elevated external Ca^{2+} can rescue synchronous release in complexin 1/2 double-knockouts but not in Syt1 knockouts. Thus, the functions of complexin and Syt1 in action potential-evoked fast neurotransmitter release are intimately related to each other yet distinct.

Biochemically investigating the relationship between the two proteins, we previously demonstrated that complexin directly binds to Syt1 even in the absence of Ca^{2+}. Because Syt1 binds to SNARE complexes weakly in the absence of Ca^{2+}, we proposed that complexin recruits Syt1 to the SNARE complex prior to Ca^{2+} influx. Recently, we examined the interaction between complexin, Syt1 and the SNARE complex using recombinant proteins. Our results indicate that Syt1 recruitment to the SNARE-driven fusion machinery by complexin is essential for vesicle exocytosis.

Syt1 bound weakly to complexin alone, but the addition of three SNARE proteins (syntaxin 1, SNAP-25 and VAMP/synaptobrevin-2) in combination, but not individually, markedly enhanced binding. Unlike full-length complexin (amino acid [aa] 1–134) and an NH2 (N)-terminally truncated complexin (aa 46–134), carboxy (C)-terminally truncated complexin s (aa 1–104 and 1–124) could not support Syt1 binding even in the presence of the SNARE complex. These results indicate that the binding of Syt1 to the C-terminal region of complexin promotes its

recruitment to the SNARE-driven fusion machinery, and that this process is crucial for Ca^{2+}-dependent vesicle exocytosis.

Theme 2. Differential localizations of GKAP/SAPAP1 isoforms in developing hippocampal neurons (Hisayo Sadamoto)

Guanylate kinase-associated protein (GKAP) and SAP90/PSD-95-associated protein 1 (SAPAP1) proteins form complexes with PSD-95 and Shank at excitatory postsynaptic sites, and are implicated in synapse formation and synaptic plasticity. GKAP/SAPAP1 proteins, which displayed different N termini, have appeared as multiple alternatively spliced isoforms. However, specific functional roles of individual isoforms still remain unclear. To understand particular functions of GKAP/SAPAP1 isoforms in formation and maintenance of synaptic connections, we here investigated expression and subcellular distributions of these isoforms in hippocampal neurons during synaptic development. First, we identified two isoforms of SAPAP1 (named as SAPAP1b and 1c) in mice hippocampus, which exhibited an alternative usage of two exons in the middle part of SAPAP1 transcript. Using primary culture of mouse hippocampal neurons and confocal microscope, we sought to examine localizations of each EGFP-tagged GKAP/SAPAP1 isoform (GKAP, SAPAP1, SAPAP1b or SAPAP1c). During synaptic maturation, GKAP/SAPAP1 isoforms were found to display differences in cluster formation at the dendritic spines. EGFP-SAPAP1 formed clusters at dendritic shafts on an early stage of synapse formation and did not change the rate of accumulation (clustering index) in mature dendritic spines at later stages. Clusters of EGFP-SAPAP1b and EGFP-SAPAP1c were also found to occur at an early stage, but tended to disappear during synaptic maturation. In contrast, GKAP clearly accumulated in dendritic spines at a later stage of synaptic maturation. These results suggest the possibility that each spliced isoform of GKAP/SAPAP1 has a specific function in synapse formation.

Theme 3. Synaptic modulation and oscillatory network modulation in odor information processing (Suguru Kobayashi)

Synchronous oscillatory activity is common in the olfactory behavior of both vertebrates and invertebrates. In the olfactory center of terrestrial animals, changes in the oscillatory frequency of the local field potential (LFP) are thought to be involved in olfaction-based behavior and olfactory memory. We study GABAergic and FMRFamidergic neuromodulation of oscillatory activity in odor information processing of the procerebrum (PC) in the land slug *Limax valentianus*. We found that GABA and FMRFamide are present in the PC and these modulatory roles are involved in the oscillatory neural network of the PC. A part of results for excitatory GABAergic and FMRFamidaergic neuromodulation are published in J. Neurophysiol. (2012) and Eur. J. Neurosci. (2010). Recent study showed the presence of cholinergic excitatory modulation for PC neurons via nicotinic ACh receptors activation (J Comp Neurol, 2014) and feedforward inhibition in the cholinergic afferents from the tentacles to the PC. Furthermore, in comprehensive analysis with *in vitro* cultured PC networks, the presence and role of biogenic amines were discussed (J Comp Neruol, 2016). We use electrophysiology and optical recording methods to understand the role of oscillatory dynamics in odor recognition and memory storage.

Grant Support: Japan Society for the Promotion of Science, Grant-in-Aid for Scientific Research.

Collaborations: With other universities.

Theme 4. Functional regulation of proteins by post-translational modifications (Ohshima)

Post-translational modifications such as ubiquitination, phosphorylation, and acetylation regulate the function of many proteins. Recently, a number of ubiquitin-like proteins (Ubl) have been identified that are covalently linked to lysine residues in target proteins. One Ubl, SUMO-1, also known as PIC1, UBL1, sentrin, GMP1, and SMT3, is an 11-kDa protein that is structurally homologous to ubiquitin. SUMO-1 modification plays an important role in altering the function of modified proteins, including transcriptional activation, nuclear localization, and decreased turnover. SUMO-1 is conjugated to proteins through a series of enzymatic steps. Initially, the ATP-dependent formation of a thioester bond between SUMO-1 and the E1 enzyme complex (SAE1/Uba2) is formed, and SUMO-1 is then transferred to the E2-conjugating enzyme Ubc9. Finally, SUMO-1 is conjugated from Ubc9 directly to a lysine residue of target proteins. The E3 ligase that conjugates SUMO-1 to target molecules in vitro and in vivo has only recently been identified. One group of such E3 ligases, protein inhibitor of activated STAT (PIAS) family proteins homologous to the yeast Siz family protein, has a conserved RING-finger domain, which regulates transactivation of many transcription factors by conjugating SUMO-1. In order to understand the molecular mechanisms by which transcriptional regulation through SUMO-1 modification, we focus the transcription (co)factors involving in cell growth, differentiation, immortalization and attempt to define the biological significance of sumoylation in carcinogenesis.

Theme 5. Analyzing the molecular mechanisms by which human T-cell leukemia virus type-1 (HTLV-1) infection is the cause of morbidity and mortality in adult T-cell leukemia (ATL) (Mukai and Ohshima).

Human T-cell leukemia virus type 1 (HTLV-1), a human retrovirus, is the causative agent of adult T-cell leukemia (ATL), an aggressive malignancy of $CD4^+$ T lymphocytes. HTLV-1 is also closely related to HTLV-1-associated myelopathy or tropical

spastic paraparesis (HAM/TSP). Although most HTLV-1-infected people are non-symptomatic, approximately 4% of patients develop ATL after a long period of latent infection (over 50 years). To date, there are few effective therapies available for ATL patients, possibly due to a lack of detailed information about the molecular mechanism of cell growth regulation by HTLV-1.

HTLV-1 basic leucine-zipper factor, HBZ, which is encoded in the complementary strand of the HTLV-1 genome, has been identified. HBZ is a nuclear protein that contains a transactivation domain and a basic leucine-zipper (bZIP) domain in its N- and C-termini, respectively. HBZ interacts with cellular bZIP proteins, in particular with the AP-1 family of transcription factors, and regulates their transcriptional activities, resulting in the control of viral gene transcription from the HTLV-1 promoter. In contrast to other viral ptotein, HBZ is constitutively expressed in all ATL patient samples because its 3'-LTR is conserved and unmethylated in ATL cells. HBZ may play a central role in the pathogenesis of ATL, however, its function has not yet been understood.

Theme 6. Analysis of Nonlinear Phenomena of Mathematical Models of Neurons (Takaaki Shirahata)

Individual neurons are capable of producing action potentials or spikes in response to external stimulation through the interactions of various ionic channels expressed on the plasma membrane of neurons. Based on data derived from the electrophysiological experiments, various mathematical models which reproduce the electrical excitability of individual neurons are developed (e.g., nonlinear ordinary differential equations). By analyzing the mathematical models in detail one can understand the dynamics of individual neurons. Modeling studies are suggested to be useful for studying drugs which are used for the therapy of channelopathy. In addition, based on the theory proposed by US mathematician and physicist, modeling studies are also expected to be useful for developing medical devices. Taking the above into account, this laboratory is investigating ordinary differential equations which describe the electrical excitability of individual neurons.

1. Research about a ghostbursting phenomenon of electrosensory pyramidal neurons in weakly electric fish
2. Research about pacemaking of RPa1 neurons in snail Helix pomatia
3. Research about the spiking activity of neocortical pyramidal neurons
4. Research about the spiking activity of vibrissa motoneurons
5. Research about the spiking activity of circadian pacemaker neurons in the suprachiasmatic nucleus
6. Research about the spiking activity of medial vestibular nucleus neurons
7. Research about the dynamics of retinal horizontal cells
8. Research about the dynamics of pseudo-plateau bursting of endocrine cells

Publications (2012~2017)

[Original papers]

2016

1. Matsuo R., Fukata R., Kumagai M., Kobayashi A., Kobayashi S., Matsuo Y. (2016) Distribution of histaminergic neurons and their modulatory effects on oscillatory activity in the olfactory center of the terrestrial slug *Limax*. *J Comp Neurol* 524, 119-35.
2. Mukai R., and Ohshima T. (2016) Enhanced stabilization of MCL1 by the human T-cell leukemia virus 1 bZIP factor is modulated by blocking the recruitment of cullin 1 to the SCF complex. *Mol. Cell. Biol.*36, 3075-3085.
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4. Shirahata, T. (2016). The Relationship of Sodium and Potassium Conductances with Dynamic States of a Mathematical Model of Electrosensory Pyramidal Neurons. *Applied Mathematics* 7(9), 819-823.
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6. Shirahata, T. (2016). Quantitative evaluations of the contribution of the excitatory ionic conductance to repetitive spiking in a mathematical model of medial vestibular nucleus neurons. *Acta Biologica Hungarica* 67(2), 215-219.
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10. Shirahata, T. (2016). The Effect of Variations in Ionic Conductance Values on the Dynamics of a Mathematical Model of Non-Spiking A-Type Horizontal Cells in the Rabbit Retina. *Applied Mathematics* 7(12), 1297-1302.

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14. Shirahata, T. (2015). Evaluation of kinetic properties of dendritic potassium current in ghostbursting model of

electrosensory neurons. *Applied Mathematics* 6(1), 128-135.
15. Shirahata, T. (2015). Numerical Simulation Analysis of a Mathematical Model of Circadian Pacemaker Neurons. *Applied Mathematics* 6(8), 1214-1219.
16. Shirahata, T. (2015). Numerical Study of a Mathematical Model of Vibrissa Motoneurons: The Relationship between Repetitive Spiking and Two Types of Sodium Conductance. *International Journal of Theoretical and Mathematical Physics* 5(3), 48-52.
17. Shirahata, T. (2015). Numerical Simulation of Bistability between Regular Bursting and Chaotic Spiking in a Mathematical Model of Snail Neurons. *International Journal of Theoretical and Mathematical Physics* 5(5), 145-150.

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18. Mukai R., and Ohshima T. (2014) HTLV-1 HBZ positively regulates the mTOR signaling pathway via inhibition of GADD34 activity in the cytoplasm. *Oncogene* 33, 2317-2328.
19. Toyama M., Aoyama H., Mukai R., Nakamura M., Yoshimura K., Okamoto M., Ohshima T., Hashimoto Y., and Baba M. (2014) A novel tetramethylnaphthalene derivative selectively inhibits adult T-cell leukemia (ATL) cells in vitro. *Anticancer Res.* 34, 1771-1778.
20. Matsuo R., Kobayashi S., Wakiya K., Yamagishi M., Fukuoka M., Ito E. (2014) The cholinergic system in the olfactory center of the terrestrial slug *Limax*. *J Comp Neurol* 522, 2951–2966.
21. Shirahata, T. (2014). Effect of sodium conductance variations on electrical behavior of a neocortical neuron model. *Acta Biologica Hungarica* 65(4), 379-384.

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26. Torikoshi K., Abe H., Matsubara T., Hirano T., Ohshima T., Murakami T., Araki M., Mima A., Iehara N., Fukatsu A., Kita T., Arai H., and Doi T. (2012) Protein inhibitor of activated STAT, PIASy, regulates alpha-smooth muscle actin expression by interacting with E12 in mesangial cells. PLoS One 7, e41186-41199.
27. Kobayashi S., Ito E. (2012) GABAergic effects on the slow oscillatory neural activities in the procerebrum of Limax valentianus. *Acta Biologica Hungarica* 63 (Suppl. 2), pp. 217–221.
28. Elekes K., Battonyai I., Kobayashi S., Ito E. (2012) Organization of the procerebrum in terrestrial pulmonates (*Helix*, *Limax*) reconsidered: cell mass layer synaptology and its serotonergic input system. *Brain Structure and Function* 218, 477-490.
29. Kobayashi, S., Matsuo, R., Sadamoto, H., Watanabe, S., and Ito, E. (2012) Excitatory effects of GABA on procerebrum neurons in a slug. *J Neurophysiol* 108, 989-998.
30. Sadamoto, H., Takahashi, H., Okada, T., Kenmoku, H., Toyota, M., Asakawa, Y. (2012) De novo sequencing and transcriptome analysis of the central nervous system of mollusc *Lymnaea stagnalis* by deep RNA sequencing. *PLoS One* 7 e42546.
31. Ito E., Otsuka E., Hama N., Aonuma H., Okada R., Hatakeyama D., Fujito Y., Kobayashi S. (2012) Memory trace in feeding neural circuitry underlying conditioned taste aversion in *Lymnaea*. *PLoS One* 7 e43151.
32. Shirahata, T. (2012). Analysis of the electrosensory pyramidal cell bursting model for weakly electric fish: Model prediction under low levels of dendritic potassium conductance. *Acta Biologica Hungarica* 63(3), 313-320.

[Books]

Mochida S., Tokumaru H. et al., (2015) “Presynaptic Terminals” Springer ISBN978-4-431-55165-2

Kagawa School of Pharmaceutical Sciences
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Center for Instrumental Analysis

Staff

Professor: Kentaro Yamaguchi, Ph. D (Apr. 2004) (LAC)
Associate Professor: Hajime Takeuchi, Ph.D. (Apr. 2013) (LPHS)
Lecturer: Kazuaki Ohara, D. Eng. (Apr. 2010)

Educational History:
Graduated from Graduate School of Tokyo University in Mar. 1992

Research

Observation of the giant molecules by means of mass spectrometry:

Mass Spectrometry (MS) has been developed and adopted to wide variety of analytical chemistry in recent years.

Although MS was basically developed for high molecular weight substances in the field of biochemistry, the measurement of huge molecules over 10k Da is still very difficult. This is caused by the ionizing problems, stability of the compounds and the existence of various impurities.

We develop some new techniques to overcome these problems by using newly equipped FT-ICR mass spectrometer.

Crystalline Sponge-Laser Desorption Ionization:

Recently, crystalline sponge (CS) method was discovered to analyze non-crystalline compounds by means of X-ray crystallography. The laser desorption ionization (LDI) mass spectrometry (MS) is first adopted to use this CS as a matrix for ionization. Since then, this new ionization method (CS-LDI MS) has been developed in this laboratory.

Publications (2012~2017)

[Original papers]

2016

1. *Tominaga, M.; Kawaguchi, T.; Ohara, K.; Yamaguchi, K.; Katagiri, K.; Itoh, T.; *Azumaya, I. (2016). Vesicle Formation of Three-dimensional Trinuclear Silver(I) Complexes Built from Tris-NHC Ligands Bearing Long Alkyl Chains. Chem. Lett., 2016, 45, 1201-1203.
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3. *Ohara, K.; Tominaga, M.; Masu, H.; Azumaya, I.; *Yamaguchi, K. (2016). Adamantane-based Bidendate Metal Complexes in Crystalline and Solution State. Anal. Sci., 2016, 32(12), 1347-1352.
4. *Kawahata, M.; Komagawa, S.; Ohara, K.; Fujita, M.; *Yamaguchi, K. (2016). High-resolution X-ray structure of methyl salicylate, a time-honored oily medicinal drug, solved by crystalline sponge method. Tetrahedron Lett., 2016, 57, 4633–4636.
5. Sawada, T.; Yamagami, M.; Ohara, K.; Yamaguchi, K.; *Fujita, M. (2016). Peptide [4]Catenane by Folding and Assembly. Angew. Chem. Int. Ed., 2016, 55, 4519-4522.
6. Ishizuka, T.; Watanabe, A.; Kotani, H.; Hong, D.; Satonaka, K.; Wada, T.; Shiota, Y.; Yoshizawa, K.; Ohara, K.; Yamaguchi, K.; Kato, S.; *Fukuzumi, S.; *Kojima, T. (2016). Homogeneous Photocatalytic Water Oxidation with a Dinuclear Co^{III}–Pyridylmethylamine Complex. Inorg. Chem., 55, 1154-1164.
7. Wang, S.; Sawada, T.; Ohara, K.; Yamaguchi, K.; *Fujita, M. (2016). Capsule–Capsule Conversion by Guest Encapsulation. Angew. Chem. Int. Ed., 55, 2063-2066.
8. *Tominaga, M.; Kawaguchi, T.; Ohara, K.; Yamaguchi, K.; Masu, H.; *Azumaya, I. (2016). Synthesis and crystal structures of twisted three-dimensional assemblies of adamantane-bridged tris-NHC ligands and Ag^{I}. CrystEngComm, 18, 266-273.

2015

1. *Ikeda, A.; Iwata, N.; Hino, S.; Mae, T.; Tsuchiya, Y.; Sugikawa, K.; Hirao, T.; Haino, T.; Ohara, K.; Yamaguchi, K. (2015). Liposome collapse resulting from an allosteric interaction between 2,6-dimethyl-β-cyclodextrins and lipids. RSC Adv., 5, 77746-77754.
2. Ohara, K.; Nakai, A.; Yamaguchi, K. (2015). Laser desorption ionization of stilbenes in crystalline sponge. Eur. J. Mass Spectrom., 21, 413-421.

2014

1. *Tominaga, M.; Yoneta, T.; Ohara, K.; Yamaguchi, K.; Itoh, T.; Minamoto, C.; *Azumaya, I. (2014). Self-Assembly of a Tetrapodal Adamantane with Carbazole Branches into Hollow Spherical Aggregates in Organic Media. Org. Lett., 16, 4622-4625.
2. *Tominaga, M.; Ukai, H.; Katagiri, K.; Ohara, K.; Yamaguchi, K.; *Azumaya, I. (2014). Tubular Structures Bearing Channels in Organic Crystals Composed of Adamantane-Based Macrocycles. Tetrahedron, 70, 2576-2581.
3. *Tominaga, M.; Iekushi, A.; Katagiri, K.; Ohara, K.; Yamaguchi, K.; *Azumaya, I. (2014). Channel-Dependent Conformations of Single-Strand Polymers in Organic Networks Composed of Tetrapodal Adamantanes with

N-heterocyclic Moieties. Tetrahedron Lett., 55, 5789-5792.

4. *Tominaga, M.; Ohara, K.; Yamaguchi, K.; *Azumaya, I. (2014). Hollow Sphere Formation from a Three-Dimensional Structure Composed of an Adamantane-Based Cage. J. Org. Chem., 79, 6738-6742.
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1. *Tominaga, M.; Iekushi, A.; Katagiri, K.; Ohara, K.; *Yamaguchi, K. (2013). Organic Crystals Bearing Both Channels and Cavities Formed from Tripodal Adamantane Molecules. Journal of Molecular Structure, 1046, 52-56.
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3. *Ohara, K.; Tominaga, M.; Azumaya, I.; *Yamaguchi, K. (2013). Solvent-dependent Assembly of Discrete and Contimuous $CoCl_2$ Adamantane-based Ligand Complexes: Observations by CSI-Mass Spectrometry. and X-ray Crystallography. Anal. Sci., 29(8), 773-776.
4. Shinozaki, Y.; Richard G.; Ogawa, K.; Yamano, A.; Ohara, K.; Yamaguchi, K.; Kawano S.; Tanaka, K.; Araki, Y.; Wada, T.; *Otsuki, J. (2013). Double Helices of a Pyridine-Appended Zinc Chlorophyll Derivative. J. Am. Chem. Soc., 135, 5262-5265.

2012

1. Ohara, K.; Yamaguchi, K. (2012). Cold-Spray Ionization Mass Spectrometric Detection of a Coordination Oligomer. Anal. Sci., 28, 635-637.

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Staff

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Associate Professor since 2005
D.Sc. in University of Tsukuba, 1994

Yoko Tominaga
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Makiko Taketoshi
Research Assistant since 2016

Research

Since the expansion of the Institute of Neuroscience, Tokushima
Research areas of the laboratory

I. Study of neural circuit mechanisms of learning and memory with optical recording methods

The primary interest of the laboratory is the neural circuit mechanisms of higher cognitive functions, such as learning and memory, in the brain. A measurement method that makes the laboratory unique in the field is an optical recording method that uses voltage-sensitive dye (VSD) with electrophysiology. As one of the leading laboratories in the use of this technique, we have been continuously developing the method since the 90s and have provided established tools to colleges throughout the world.

II. Analysis of the electrophysiological control of excitable membranes in connection with ciliary structures.

By focusing on the role of information integration in the membrane potentials of cells, we have used the model organism, paramecium, which is the simplest single-celled animal, to study the mechanisms of the membrane potential control of cilia.

Specific research aims

Area I

1. Neural circuitry mechanisms of the limbic system: Optical study.

The limbic system is a brain structure that is critical for emotion and declarative memory. The limbic system consists of many areas, including the hippocampus, amygdala, and associated cortical systems, such as the entorhinal and piriform cortices. We are analyzing the function of these circuits by visualizing neural activity with the VSD optical recording methods.

We have revealed reverberation circuits and information integration mechanisms in the deep layers of the entorhinal and piriform cortices in association with the hippocampal neural circuit (Science, 1996; Neurosci. Res., 2008) with Professor Toshio Iijima's group at Tohoku University. In addition, we have found that neuronal signals from layer III of the medial entorhinal cortex are critical for temporal association memory formation (Science, 2011) with Professor Susumu Tonegawa's laboratory at the Picower center for learning and memory at MIT. We have also revealed information integration processes in the entorhinal cortex (Eur. J. Neurosci., 2007) with Dr. Riichi Kajiwara and Dr. Ichiro Takashima's group at AIST Japan. We showed that the D-current plays an important role in the integration of neural activity in the entorhinal cortex in collaboration with Dr. Riichi Kajiwara (Japan Society for Neuroscience, 2012; Society for Neuroscience, 2012; supported by KAKENHI).

2. Development of an optical measurement microscope: stimulation pattern with a confocal microscope system and a new optical measurement.

The optical recording method with VSD requires high-speed and low-noise imaging. This requires new special optics. We have been developing special optics that meet these requirements (J. Neurosci. Methods, 2000; now commercially available as THT-microscope, BrainVision).

We have also developed special new ultra-high-speed and low-noise confocal optics (submitted; supported by JST tansaku, A-STEP). We have developed a microscope that allows us to conduct light stimulus patterns to the neural networks (SFN abstr., 2011). Recently, we have started a project to develop a special chamber that is suitable for these experiments (Supported by JST A-STEP, 2012-2013).

3. Mechanisms of late-onset brain dysfunctions caused by early transient exposure to chemicals and drugs.

There are several lines of evidence that indicate that the early transient exposure to certain chemicals during brain development results in the malfunctioning of cognitive function in adulthood. The neural mechanisms of these effects are largely unknown. We are evaluating these neural mechanisms with our optical recording methods as part of the research team that is supported by the Ministry of Health, Labour and Welfare (2008-, 2015-).

We have shown that the administration of valproic acid, which is the first-line drug used in the treatment of epilepsy, during pregnancy causes a collapse of the balance of excitation and inhibition in children born to these mothers (Japanese Society of Toxicology, 2012). This study is joint research that is being conducted with Kentaro Tanemura sensei of Tohoku University, Dr. Yoshikazu Nakajima of Nara Institute of Science and Technology, and the teacher Katsuhide Igarashi of the Japan Institute of Health Sciences. We organized a symposium at the Japan Neuroscience Society in Kyoto in June 2013.

4. Study of the Application of optical measurement methods to test

ES cell function.

This study was initiated in 2012 and is intended to use the optical recording method with VSD to characterize cells that are differentiated from human ES cells. This is joint research that is being conducted with Prof. Katsunori Sasaki, Shinshu University [supported by KAKENHI (A)].

5. Visualization of cell-specific membrane potential responses by the introduction of voltage sensitive fluorescent protein (VSFP), which is a new membrane potential-sensitive protein.

In collaboration with Dr. Thomas Knopfel at RIKEN BSI, we have succeeded in detecting optical signal-specific hippocampal pyramidal cells by introducing a new VSFP from 2012. The detection of cell-specific signals are made possible in specimens in vivo by the further development of this technique.

6. Detection and use of the optical signals from neural excitation with a polarized light microscope.

This is joint research that is being conducted with Dr. Tomomi Tani and Dr. Oldenburg of Woods Hole MBL. In this study, we aim to detect changes in nerve optical properties, such as polarization, that are caused by nerve excitation. In March of 2013, we will visit the MBL for this purpose.

7. Studies of the mechanisms of regulation by a variety of factors and the neural responses of hippocampal neural synapses.

We are collaborating with various laboratories to apply our method in order to examine the neural pathologies of diseases, such as Alzheimer's disease, and other factors (J. Neurosci., 1996; Neurosci. Letters, 1997; J. Neurosci, 2002; PNAS, 2004; Neuropharmacol., 2005).

8. Regulation mechanisms of neural activity by inhibitory synapses in the hippocampus.

The unique feature of VSD imaging compared to other biological imaging methods is that it can measure hyperpolarization and, thus, inhibitory neural responses. From this point of view, we found depolarizing GABA responses in area CA1 in response to tetanic stimulation (J. Neurophysiol., 2002; Pfluger's Arch., 2010). In addition, we found perisomatic inhibitory actions with feedforward inhibition (Neurosci. Res., 2009).

Area II

1. Physiological studies of osmoregulatory mechanisms and contractile vacuoles of Paramecium.

For the first time, we have applied electrophysiological methods to the study of the Paramecium organelles, the contractile vacuoles, and have revealed the membrane dynamics that are involved in this periodic activity (J. Exp. Biol., 1997a, b; 1998a, b; J. Cell Sci., 1999; J. Exp. Biol., 2005).

2. Physiological studies of membrane proteins and cilia of paramecium response mechanisms.

The use of recent techniques of RNA interference knockdown in combination with the whole genome project of the Paramecium has enabled us to knock down particular proteins that are associated with cilia disease (so-called ciliopathy). We have found that the absence of some molecules that have been thought to be structural proteins induces behavioral changes. By applying electrophysiological methods to this mutant, we have examined the relationship of that behavior and the membrane responses and found that some of these "structural proteins" are actually involved in membrane potential-mediated signal transduction (e.g., Eukary. Cell, 2012). This is joint research that is being conducted with Prof. Hori of Yamaguchi University.

Publications (2012~2017)

[Original papers]

*Corresponding author

2016

1. Yoshimura, H., Sugai, T., Kato, N., Tominaga, T., Tominaga, Y., Hasegawa, T., Yao, C., and Akamatsu, T. Interplay between non-NMDA and NMDA receptor activation during oscillatory wave propagation: Analyses of caffeine-induced oscillations in the visual cortex of rats. ***Neural Networks***. 79:141-149 (2016) DOI: 10.1016/j.neunet.2016.03.012
2. *Tominaga T and Tominaga Y (2016). Paired burst stimulation causes GABAA receptor-dependent spike firing facilitation in CA1 of rat hippocampal slices. *Front. Cell. Neurosci.* **10**:9. doi: 10.3389/fncel.2016.00009

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4. Takashi Tominaga[CA], Riichi Kajiwara, and Yoko Tominaga (2013) VSD imaging method of ex vivo brain preparation *Journal of Neuroscience and Neuroengineering* 2, 211-219 (2013) *[Featured Article に採用]*
5. Tominaga T[CA] and Tominaga Y. (2013) A new non-scanning confocal microscopy module for functional voltage- sensitive dye and Ca2+ imaging of neuronal circuit activity Journal of Neurophysiology J Neurophysiol 110, 553-561; published ahead of print April 24, [Also featured as Key Scientific Articles on Global Medical Discovery]

2012

1. Kutomi, O., Hori, M., Ishida, M., Tominaga, T., Kamachi, H., Koll, F., Cohen, J., Yamada N and Noguchi M. (2012). Outer Dynein Arm Light Chain 1 Is Essential for Controlling the Ciliary Response to Cyclic AMP in Paramecium tetraurelia. Eukaryotic cell, 11(5), 645–653. doi:10.1128/EC.0527