



protein within them, securing selective toxicity is often difficult. Furthermore, if target can be determined, it facilitates comprehension of action mechanism in antimicrobial agent and will be useful information in development.

However, determining targets of antibiotics is regarded as a difficult task. For novel antibacterial drug except the existing antibiotics derivatives, development of drugs is considered without determining targets. Lately, we have established the innovative method to solve such problems. (Japan Patent Application No.2011-124011 ) The method applies methodologies of biochemistry and genetics and it enables determination of the target in a short period of time. We apply this method to Kaikosin that is discovered as a novel antibiotic and determine targets successfully. (Unpublished data) Please contact us if you have antibiotics that targets are unknown.

Sep 11<sup>th</sup>, 2012

## **7. Recommendation of “Silkworm Drug Discovery”**

I would like to propose an innovative drug discovery concept named “Silkworm Drug Discovery” method. This method adopts silkworms as disease-model animals to find out lead compounds that possess therapeutic effect and introduce it to pharmaceutical firms. Specifically, the following procedures are taken.

1. Building disease model using silkworm
2. Search for therapeutically active substance
3. Validation of the therapeutic effect with mouse models

As you see the above, it is not the case that using silkworms for ingredients used for making medicines nor mass-producing proteins. This is illustrated in the following example.

1. Building disease models using silkworm

Silkworms possess various organs similar to Homo sapiens' ones. In the body of silkworms, there is blood, brain, gastrointestinal tract, heart, kidney, muscle and nervous system. It is not easy to list objects Homo sapiens possess and silkworms do not. Therefore, it is possible to build models for Homo sapiens using silkworms as test animal. With silkworm experimentation, we have developed bacterial infection, fungal infections and viral infections models. And furthermore, we built a diabetes model successfully. I understand some may wonder why silkworm can be a test animal for diabetes model. It will be displayed under a separate section. In order to build silkworm experimental disease model, we try to achieve following points, to find prominent symptoms, such as death and to detect therapeutic effect in both silkworm and homo-sapiens. With the care of these points, we conduct a drug seeds search.

## 2. Search for therapeutic active substances

The objective of "Silkworm Drug Discovery" is to discover the seeds of a new drug from natural or compounds library, using therapeutic effect that is against disease model of silkworm as an indicator. Generally, in the development of pharmaceutical products, the evaluation of the therapeutic effect by animal studies is tested for compounds which are searched in vitro test. An innovative aspect of the discovery silkworm drug is to search compounds using therapeutic effect as an indicator. In the evaluation system using mammals, it is hard to search the drug candidate compounds using a large number of samples because of cost and ethical issues. However, silkworms solve the problems. We are focusing on the culture supernatant of the microorganism as a natural product. Microorganisms produced various substances as secondary metabolites. "Silkworm Drug Discovery" finds substance that indicates the therapeutic effect with silkworm as test animals. In the case of natural products, it is necessary to purify the substances using an activity in the fractions as an indicator and determine the structure. It goes without saying that efforts are required in this regard. In recent years, we are able to take advantage of a variety of compound libraries. Compound library is

composed from compounds that synthesis methods have been established. Among the libraries, searching for new drug candidate compounds with new evaluation methods is widely used. "Silkworm Drug Discovery" searches for a compound from library using therapeutic effect as an indicator. This method requires modification of found compound with organic and chemical techniques and finds novel structured derivatives. At this stage, the cooperation of organic chemists is required.

### 3. Verification of therapeutic effects in mouse disease model

The therapeutic effect assessment of medicine is performed using mammals such as mice. Therefore, even if a therapeutic effect is detected from silkworm case, it is not possible to deliver the result to pharmaceutical companies as pharmaceutical seeds without effect confirmed with experiment using mammals. For candidate compounds showed a therapeutic effect in silkworm, a validation is required in the mouse model. There are very few compounds that show therapeutic effect on silkworm and do not on mouse from our study of antibiotics so far. Therefore, the efficiency of animal experiments can be improved with silkworms as test animals. In the development of pharmaceutical products, a variety of toxicity tests are performed against candidate compounds that show therapeutic effect in mammal. This is called pre-clinical studies. Additionally, clinical trials in humans are necessary before Ministry of Health, Labour and Welfare approves medicines. Development of pharmaceutical products requires a long time, I hope "Silkworm Drug Discovery" performs well in the searching stage before using mammals.

Aug 26, 2011

### **6. The elements human bodies have and silkworms lack for**

Do you think it is too much to say "silkworms are crawling human beings"? It is not easy to name internal organs that human bodies have but silkworms lack for. Silkworms have many common aspects with human beings. Some say "humanity" should be the original characteristics of human beings

what do you think?

Let's think of elements that are purely original for human bodies from biological point of view. Firstly, the blood of silkworm is transparent because it lacks for a red blood corpuscle (erythrocyte). Secondly, invertebrate animals including silkworms do not own antibody genes that are essential for acquired immunity in vertebrate animals. Silkworms do not have genes encoding antibodies. Therefore, silkworms don't have immune system based on an antigen-antibody reaction. Lastly, silkworms lack for tissues whose function is equal to bones for human bodies that mean we cannot create disease model of osteoporosis using silkworms. Now you see it is not impossible to name some organs, tissues that are characteristics for human beings and silkworms lack for.

It is rather easy to find common factors between human bodies and silkworms. Silkworms have brains, neural systems, muscles, and organs whose functions are equal to human's heart, livers, kidneys, and digestive tracts in human bodies, and we can create various models for human beings' diseases. We are building up infectious models using silkworms with germs/virus, and trying to study disease models of diabetes and liver diseases. In principle, most of the diseases can be modeled using silkworms. I expect the disease models on silkworms as most useful for producing new effective drugs.

Jul 14<sup>th</sup>, 2011

## **5. Drugs, effective or non effective**

When discovering new antibacterial drugs, it is very important to confirm the curing effect. It is quite easy to find a chemical substance, which inhibit the growth of bacteria in a test tube, among both of naturally originated sources, and of artificially made synthesis. However, few substances can show actual effect on curing model animals, infected with diseases. It is because most of the substances have problems on pharmacokinetics in animal bodies; let's take drug for septisema as an example. To cure the disease effectively, drug needs to be absorbed through intestinal tract. If the amount of molecule of a substance exceeds 500 , the substance is hard to be absorbed through

intestinal tract. Moreover, drug transferred through intestinal tract in blood vessels needs to be “distributed” in blood. Organic synthesized substances are mostly hydrophobic ( tend not to mix with water well), so they get dispersed in organs and extinguish swiftly after injection from blood. The drugs must evade the “metabolism” by P450, after arriving at the liver through intestinal tract, because most metabolized substances do not show activity for curing. Kidneys and other similar organs with “excretion” function remove substances in blood out of the body. Those substances, evacuated instantly out of blood, cannot act on bacteria in blood.

As described above, pharmacokinetics of drug depend on 4 elements, such as “Absorption”, “Distribution”, “Metabolism”, “Excretion”, named ADME for their capitals. Effective drug must have good function of ADME. Plus, having no Toxicity is essential for an effective drug. These characters are named ADMET, and finding new substances with good nature regarding ADMET is the key for discovering effective drug.

Generally, ADMET of a candidate substance for new drugs were examined with mammals, such as mice. However, to sacrifice mammals is now often discussed as more problematic, not only because of high cost, but also because of ethical reasons. We are suggesting making most use of a silkworm as a new model animal, to examine ADMET of drug candidate.

We regard it possible to use silkworms as pre-model animals, before testing drugs on mammals such as mice, the ethical problem with sacrificing mammals can be solved. We have actually succeeded to discover a new antibiotic, named “Kaikoshin”, which is found to be effective against disease model on mice. We, as a small researching team have gained such a success, thanks to using silkworms as model animals.

The issue with ADMET is important not only for antimicrobial drug, but also for all the medicines. I think it possible, that in near future, silkworms would be more of use for quite a few fields of diseases, to examine effective drugs.

Jul 7<sup>th</sup>, 2011

#### 4. Why silkworm?

It has been nearly 10 years since we first suggested using silkworm as model animal for testing the curing effect of medicine (Kaito *et al. Microb Pathog*, 32, 183-190, (2002)). Since then, I have been questioned for the reason of selecting silkworm as a model animal quite a few times. Here I shall explain why silkworm has been selected for our model animal.

In 2000, I was studying genetics and biochemistry of DNA replication in *Staphylococcus aureus*. I always tried to emphasize the difference and uniqueness of the study on *Staphylococcus aureus* from those on *Escherichia coli*. Pathogenicity is the most characteristic feature of *Staphylococcus aureus*. The most common way of measuring the pathogenicity of bacteria is analysis of diseases after injection on animals, usually mammals, like mice. However, mammals as model animals require to be proved to be free from contamination with bacteria (called SPF animals if proved to be clean). Those infection tests using SPF animals are quite expensive, and thus practically not possible in study in university. I searched for the appropriate animals suitable for testing pathogenicity - frogs, guppies (popular tropical fishes), earthworms, scorpions (they are largely grown as food in China) were tested with injection of *Staphylococcus aureus*, and silkworms were finally found to be most suitable for testing. The most unique point of silkworm is that we can obtain so many of them with low cost, all round the year. For this point, I regard silkworm as the best model animal for testing in study in Japan. I was not sure at the beginning, if I could really with silkworm, an insect, test the infectious effect, or examine the curing effect of drugs against diseases, but it was actually found that both "infection" by pathogenic agent and "curing" by antibiotics were successfully observed. Also, though not had been expected at the beginning, the amount of antibiotic required for gaining therapeutic effects (ED<sub>50</sub>) on silkworm was found to be quite equal to those on mammals.

Many researchers in pharmaceutical companies point it out, that the testing results on model animals like mice, and those on clinical situation are not analogous. Some of those researchers claim that using silkworm as a model

animal is out of question. However, it is actually observed that effecting results on human being and on silkworm match on several aspects; in silkworm, we can observe the initial phase of drug metabolism by the reaction with P450 and following conjugation reaction, just like in mammals. A silkworm has organs functioning like liver and kidney.

Recently it is getting rather difficult to sacrifice a large numbers of mammals to evaluate therapeutic effects of drug candidates for reasons of animal welfare. If we decide to use silkworms as new model animals, we can remarkably reduce the numbers of mammals required for drug development.

I started using silkworms as model animals because I could examine the pathogenicity of bacteria less expensively, but quite a few variations of diseases are now established with silkworms as model animals. Besides infection models with bacteria, infection models with fungus, and with virus (silkworms can be infected with baculovirus, with which all insects can be commonly infected) have been established in my laboratory. Also we have succeeded to make hyperglycemia model by adding glucose to diet for silkworms (Mastumoto *et al.* PLoS ONE 6(3): e18292 (2011)). I expect to find new compounds, effective for curing diabetes with this model. Additionally, hepatic disease model can be made using a silkworm. A silkworm has innate immune system, common with what a human being has. As mentioned above, a silkworm has most of the organs, whose functions are correspondent with those of human beings'; a silkworm has a brain, a nervous system, muscle, alimentary canal, heart, and an organ called Malpighian tubele, whose mechanism is just like kidney for human beings. Thus, principally, we can make quite a few models with silkworm of diseases for human beings. We are attempting to discover new drug candidates by use of disease models with silkworms.

Jul 7<sup>th</sup>, 2011

### **3. An index for pharmacokinetics of antibacterial substances**

Generally, the antibacterial activity of compounds are expressed with MIC; Minimum Inhibitory Concentration, a concentration of the drug to terminate

the growth of the bacteria in the test tube. The less the MIC value is, the more powerful the antibacterial activity of the material. Traditional drug discovery method has been focused to find compounds with the least MIC value. However, even with little MIC value, the compounds with problems on pharmacokinetics in animal bodies cannot exhibit any therapeutic effects. It is generally known that less than 1 % of the compounds that are examined as with antibacterial effects in test tubes, can really show therapeutic effects in experiments on animals. Most of the compounds have problems on pharmacokinetics in animal bodies.

The therapeutic effects of drugs are estimated with values to show how much of the compounds is necessary to gain the therapeutic effects, which is called  $ED_{50}$ .  $ED_{50}$  means amount of the drug needed for curing 50 % population of animals. If pharmacokinetics of a compound is ideal,  $ED_{50}$  matches MIC. The less a ratio between  $ED_{50}$  and MIC is, more effective the pharmacokinetics in animal bodies. On the other hand, the larger the ratio value is, the more problem the compound has, with pharmacokinetics in animal bodies. We call this ratio as "An index for pharmacokinetics of antibacterial substances".

We are searching for compounds with less value of an index for pharmacokinetics of antibacterial substances using silkworm infection model. Recently we succeeded discovering a new antibiotic "Kaikosin", with therapeutic activity. I see the small value of an index for pharmacokinetics of antibacterial substances is necessary for an antibacterial drug. Surprisingly many of the drugs have nearly the similar values on indexes for pharmacokinetics of antibacterial substances on silkworms and on mammals. Antibacterial drugs, which are used for clinical purposes generally show small value if tested on silkworms.

Little attention has been paid on an index for pharmacokinetics of antibacterial substances for discovery of antibacterial drugs. It is because obtaining the  $ED_{50}$  amount value which shows the therapeutic effects of the candidate compound is not easy. With silkworm as model animal the value can be obtained with much less costs, with less ethical problems, compared with mammal animals such as mice. MIC, on the other hand, can be easily obtained

in laboratory with general experimental equipment for microbiology. Therefore, once the value of  $ED_{50}$  is obtained, an index of pharmacokinetics in animal bodies is easily calculated. Traditional method of antibacterial drug discovery has been based on MIC, but now I shall suggest setting the index for pharmacokinetics of antibacterial substances-is quite important.

Jul 7<sup>th</sup>,2011

## **2. Method based upon the idea of genome-drug discovery**

~the epoch-making method to determine a target of a drug candidate ~

### Need for establishment of new methodology for drug discovery; against infectious diseases

It is necessary to find new antibiotics, to get radical control over diseases caused by bacteria, resistant with existing drugs. However, discovering new antibiotics with unique constructions is laborious and expensive, thus hard to be carried out. Looking in the drug-discovering history in these 50 years, you will see how hard it is, with how little varieties of new antimicrobial medicines were found, either of naturally originated sources, or of artificially made synthesis. This situation indicates that we need to build up a brand new original method for drug discovery, to achieve the discovery of unique antimicrobial medicines.

### The traditional methods of genome pharmaceutical sciences for antimicrobial drug discovery

In this quarter of a century, it has been tackled in all over the world, developing new antimicrobial drug based upon the idea of genome drug development. It is a drug design method of focusing the genome of bacteria, with which a certain protein is initially chosen to be targeted, and then inhibitors against the target should be searched for. However, the method could not provide the expected results, as I discuss the reason in following. Generally, targeted protein of antimicrobial drug is essential for growth of bacteria. Many

researchers have tried with many of the methods, to define the genes, encoding essentially required proteins for pathogen to grow. We too, with combined researching with pharmaceutical companies, devoted our energy to study and defined more than 100 of *Staphylococcus aureus* genes essential for the growth of bacteria by defining genes more than 1000 of temperature-sensitive mutants. Considering that the estimated number of essential genes for bacteria to grow is said to be 200, we can say to have defined about half of the essential genes for *Staphylococcus aureus*. The proteins, encoded by the defined genes, were firstly expressed in *Escherichia coli*, then purified as recombinant proteins.

With large-scale screening by robots (called HTS; High Throughput Screening), inhibitors which inhibited the activities of the recombinant proteins were selected from chemical libraries which contain compounds of hundreds-thousands, or in some cases, of more than a million. Such method was practiced by many of the pharmaceutical companies in the world, including us. As had been predicted, most of the chemical compounds, actually sorted, did not affect growth of bacteria. It was mainly because most of the chemical compounds cannot pass through the cell membrane of bacteria.

Some of the sorted chemical compounds were found to be with antibacterial activity. However, looking into the mechanism of the activity of those, no evidence was found that such activity was gained because these compounds specifically inhibited the activity of the protein, although had been expected to do so.

Among quite a few methods for investigating the action mechanism of antibacterial compounds, the most common way is monitoring macro molecule synthesis in bacteria cells, using radio-labeled precursors; thymidine for DNA, uridine for RNA, amino acids for proteins, and N-acetylglucosamine (GlcNAc) for peptidoglycan, are precursors for each macro molecule. Taking an example, we found a reagent which inhibited the activity of a protein necessary for DNA replication, however, the reagent prevented bacteria growth without inhibition of DNA synthesis in bacteria cells.

To be honest, I had not expected such result to come up that time.

The importance of identifying the target protein of candidate compound for development of new antibacterial drug

As mentined above, it is quite difficult by traditional methods, which determine a target at first followed by HTS, to discover new antibacterial drugs which specifically inhibit the activities of the essential proteins. As a solution, I suggest a new strategy where we identify the target proteins for reagents having antibacterial activities.

If it is possible to identify targeted protein for each of the antibacterial substances, which would be potential candidate for new drugs- either in future, or being undergoing development already-, the aim of genome-drug discovery would be achieved, since the targets are already defined beforehand of the development.

However, defining the target of antibacterial compounds has been seen to be quite of a difficulty in general.

Now we succeeded to build a new methodology to resolve the difficulty (Pat. No. 2011-124011). We have started to confirm if one can serve in practice for antibacterial drug discovery, with cooperation of pharmaceutical companies. I would be most pleased to keep you informed of our study results.

Jun 30<sup>th</sup>, 2011

## **1. Discovery of “Kaikosin”**

Recently, we discovered new antibiotic produced by soil bacteria, and named it as “Kaikosin”. It is proved to be effective against golden staph (Stapylococcus aureus), known as MRSA, notorious to bring a nosocomial infection. The name of Kaikosin derives from “Kaiko” which means silkworm in Japanese. We purified Kaikosin from the culture supernatant by measuring the therapeutic effects of each fraction using silkworm infection model.

Traditional method for discovering new antibiotic was carried out by measuring the concentration of each antibiotic necessary for terminating the growth of pathogenic microbes in a test tube, which is called MIC (Minimum Inhibitory Concentration). Instead of that, we used silkworm as model animal to measure the actual effectiveness on curing animals. Development of new strategy for discovering new antibiotics by measuring therapeutic effects is quite a unique point in our study.

Jun 23<sup>rd</sup>, 2011