(Form4-2)

JOINT RESEARCH PROJECT

FINAL REPORT

For Japan-Korea Joint Research Project

AREA	1. Mathematics & Physics 2. Chemistry & Material Science ③ Biology 4. Informatics & Mechatronics 5. Geo-Science & Space Science 6. Medical Science 7. Humanities & Social Sciences

1. **Research Title:**

Behavior and Toxicity of Polycyclic Aromatic Hydrocarbons in East Asia

2. **Term of Research:** From July 1, 2009 To June 30, 2011

3. **Total Budget**

a. Financial Support by JSPS: Total amount: 2,400 thousand yen

1st Year 1,000 thousand yen 2nd Year 1,200 thousand yen

3rd Year 200 thousand yen

b. Other Financial Support : Total amount: 0 thousand yen

4. **Project Organization**

a. Japanese Principal Researcher	
Name	Akira Toriba
Institution / Department	Institute of Medical, Pharmaceutical and Health Sciences/ Graduate School of Natural Science and Technology, Kanazawa University
Position	Associate Professor
b. Korean Principal Researcher	
Name	Hae Young Chung
Institution / Department	Department of Pharmacy, College of Pharmacy, Pusan National University
Position	Professor

c. List of Japanese-side Participants (Except for Principal Researcher)

Name	Institution/Department	Position
Takuya Kawanishi	Institute of Science and Engineering, School of Natural System	Associate Professor
Kazuichi Hayakawa	Institute of Medical, Pharmaceutical and Health Sciences	Professor
Takayuki Kameda	Institute of Medical, Pharmaceutical and Health Sciences	Assistant Professor
Ning Tang	Institute of Medical, Pharmaceutical and Health Sciences	Assistant Professor
Nobuo Suzuki	Institute of Nature of and Environmental Technology	Associate Professor
Chun-Sang Hong	Institute of Medical, Pharmaceutical and Health Sciences	P.D.
Ying Li	Institute of Medical, Pharmaceutical and Health Sciences	P.D.
Tomoyasu Tsukamoto	Institute of Medical, Pharmaceutical and Health Sciences	P.D.
Maromu Yamada	Center of Innovation	P.D.
Kanae Bekki	Graduate School of Natural Sci. and Tech.	Ph.D. Course
Hossam F. M. Nassar	Graduate School of Natural Sci. and Tech.	Ph.D. Course
Pham Chau Thuy	Graduate School of Natural Sci. and Tech.	Ph.D. Course
Chuesaard Thanyarat	Graduate School of Natural Sci. and Tech.	Ph.D. Course
Morio Yoshita	Graduate School of Natural Sci. and Tech.	M.S. Course
Kou Miyakawa	Graduate School of Natural Sci. and Tech.	M.S. Course
Chizuru Kinoshita	Graduate School of Natural Sci. and Tech.	M.S. Course
Hisatoshi Nakase	Graduate School of Natural Sci. and Tech.	M.S. Course
Jun Nakano	Graduate School of Natural Sci. and Tech.	M.S. Course
Akihiko Izaki	Graduate School of Natural Sci. and Tech.	M.S. Course
Michiya Tatematsu	Graduate School of Natural Sci. and Tech.	M.S. Course
Kimi Kawabe	Graduate School of Natural Sci. and Tech.	M.S. Course
Nattapoon Nunto	Graduate School of Natural Sci. and Tech.	Research Scholar (M.S. Course)
Ruibo Li	Graduate School of Natural Sci. and Tech.	Research Scholar (Ph.D. Course)

d. List of Korean-side Participants (Except for Principal Researcher)

Name	Institution/Department	Position
Sang Woon Chung	Department of Pharmacy, College of Pharmacy	P.D.
Eun Kyeong Lee	Department of Pharmacy, College of Pharmacy	P.D.
Hyoung-Sam Heo	Department of Pharmacy, College of Pharmacy	P.D.
Jehun Choi	Department of Pharmacy, College of Pharmacy	M.S. Course
Young Mi Ha	Department of Pharmacy, College of Pharmacy	M.S. Course
Ji Min Kim	Department of Pharmacy, College of Pharmacy	M.S. Course
Eunkyue Choi	Department of Pharmacy, College of Pharmacy	M.S. Course
Yu Kyeong Han	Department of Pharmacy, College of Pharmacy	M.S. Course
Hyun Jung Kim	Department of Pharmacy, College of Pharmacy	M.S. Course

5. Number of Exchanges during the Final Fiscal Year*

a. from Japan to Korea *Japanese fiscal year begins April 1.

Name	Home Institution	Duration	Host Institution
For Final Fiscal Year(FY2011) Total: 0 persons		For Final Fiscal Year(FY2011) Total: 0 man-days	
Numbers of Exchanges during the past fiscal years			
FY2009: Total 7 persons FY2010: Total 6 persons			

b. from Korea to Japan

Name	Home Institution	Duration	Host Institution
For Final Fiscal Year(FY2011) Total: 0 persons		For Final Fiscal Year(FY2011) Total: 0 man-days	
Numbers of Exchanges during the past fiscal years			
FY2009: Total 6 persons FY2010: Total 5 persons			

6. Objective of Research

The transportation of oil increases rapidly with the increase of oil demand in the world and the danger of marine pollution due to the oil outflow accident rises in East Asia. Crude oil contains various hydrocarbons. Among them, polycyclic aromatic hydrocarbons (PAHs) have carcinogenesity/mutagenicity and those are considered to have the influence on the animal in the polluted region. However, there is no report of the relationship between morphological abnormalities in animals such as fishes and shellfishes and exposure to PAHs. For example, the spinal column curve of the flatfish fry hatched in the polluted seawater was observed, but the reason for this symptom is still unclear [*Water Res.*, **40**, 981 (2006)].

On the other hand, PAHs are generated when the fossil fuel is burnt and become stronger mutagenic nitropolycyclic aromatic hydrocarbons (NPAHs) through the reaction with nitrogen in the air. Combustion particulates containing PAHs and NPAHs cause serious urban air pollution all over the world and are considered as a cause of lung cancer in human. In China, the atmospheric concentrations of PAHs and NPAHs were 20-300 times higher than those in Japan, because a large amount of coal is used for heating in winter in China [*Atmos. Environ.*, **39**, 5817 (2005); *Environ. Foreisic Sci.*, **8**, 165 (2007) etc.]. In addition, they are long-range transported to South Korea and Japan [*Atmos. Environ.*, **41**, 2710 (2007)] and form new toxic species in the presence of sun light and Asian dust. The resulted pollutants are considered to cause several diseases such as asthma and cardiac infarction.

Based on the current state of serious PAH/NPAH pollution of East Asia described above, the collaboration study by special researchers in Japan-South Korea focused on the following purposes:

(1) Behaviors of PAHs/NPAHs in East Asian area and their chemical/metabolic activation (Japanese side), and (2) Redox signaling pathway and aging-accelerating activity of PAHs metabolites. (Korean side).

For the first purpose, we studied on the distribution and transportation of PAHs/NPAHs and their chemical/biological activation to have their carcinogenicity/mutagenicity and endocrine disruption. Second, the novel toxicities for hyroxides and quinones of PAHs/NPAHs were suggested. We explored the formation of reactive oxygen species (ROS), redox signaling pathway and aging-accelerating actions of their hydroxides and quinones to have strong toxicity and endocrine disrupting activity. The collaborative project not only provided the basic understanding of environmental effects of PAHs/NPAHs and their analogues, but also suggested molecular mechanism of redox signaling by their metabolites.

7. Methodology

1. Collection of airborne particulates in Asian countries and analysis of PAHs/NPAHs

Airborne particulates were collected at a site in Shenyang, China in 2001 and 2007, and two sites in Hanoi, Vietnam in summer season of 2010 using high-volume air samplers at a flow rate of 1000 L/min or low-volume air samplers at a flow rate of 28.3L/min. Airborne particulates were collected on quartz fiber filters. The filters were weighed and then stored in a refrigerator until use. Both PAHs and NPAHs were extracted ultrasonically twice with benzene/ethanol (3:1, v/v), and the extract was washed with sodium hydroxide solution, sulfuric acid solution and water. The organic phase was evaporated to dryness, and then the residue was dissolved in ethanol. The nine PAHs in the extracts from the collected airborne particulates were determined by using high-performance liquid chromatography (HPLC) with fluorescence detection. Eleven NPAHs were determined by HPLC with chemiluminescence detection.

2. Transcriptional activation via nuclear receptors and gene expression patterns of drug-metabolizing enzymes

Aryl hydrocarbon receptor (AhR) binding activity using the recombinant rat hepatoma cell and thyroid receptor β (TRβ), and estrogen receptor (ER) binding activities using the recombinant human breast cancer cells with the receptors regulated luciferase gene construct were measured in 25 PAH derivatives and environmental samples (i.e., airborne particles and indoor dusts) in order to detect endocrine disruption (ED) potencies. All test compounds were evaluated in terms of agonist, antagonist, and potentiating activities. Luciferase activities of the tested compounds corresponding EC_5, EC_{20}, and EC_{50} (5, 20, and 50% Effective Concentrations) of 2,3,7,8-TCDD were used to define EC_5, EC_{20}, and EC_{50} concentrations of each compound. In addition, binding activities of the samples with transthyretin (TTR) were investigated by radioisotope method. mRNA expression of phase I and phase II drug-metabolizing enzymes was measured by real-time PCR.

3. Measurement of endocrine-disrupting activities by the yeast two-hybrid assay and binding affinity assay

Estrogenic and antiestrogenic activities of quinoid PAHs (PAHQs), ketone PAHs and hydroxylated nitropyrenes (OHNPs) were evaluated by the yeast two-hybrid assay using yeast cells expressing estrogen receptor-α (ERα). Relative effective potencies of estrogenic and antiestrogenic activities were calculated as the inverse values of the relative concentration of the test compound that gave the same activities of E_2 and 4-hydroxytamoxifen, respectively. Binding affinity of the tested compounds to ERα was assayed by the polarized fluorescence method using Fluormone™ ES2.

4. Measurement of the viability of A549 cells, H_2O_2 production and covalent binding to thiol groups

Cell survival was quantified by the colorimetric assay based on the conversion of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to MTT-formazan derivative by mitochondrial enzymes in viable cells. At least three independent experiments were performed. The cytotoxic effect of quinoid PAHs, on the other hand, was assessed as percent cell viability where vehicle-treated cells were taken as 100% viable.

The concentration of H_2O_2 was determined by means of ferrous iron oxidation in xylenol orange (FOX assay). Cells in 96 well plates, pretreated with buthione-[*R,S*]-sulfoximine for glutathione depletion and 3-amino-1,2,4-triazole for catalase inactivation. After preincubation, cells were treated with each quinoid PAHs and incubated with Krebs-Ringer phosphate buffer (pH 7.4) containing 5 mM glucose. The reactant was mixed with FOX working reagent, and the absorbance at 540 nm was read in a plate reader.

For the measurement of thiol group (DTT assay), the test solution was mixed with Tris-HCl/EDTA buffer and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). Absorbance of each resulting mixture was measured at 412 nm against a blank solution to determine the content of the thiol group.