Chapter 10

FURTHER ENHANCEMENT OF THE EPR EFFECT

The vascular density of rapidly growing tumors such as hepatocellular carcinoma (primary liver cancer) and renal cell carcinoma is usually quite high, whereas that of other tumors, such as pancreatic and prostatic cancer, and metastatic liver cancers, is low. Such hypovascular tumors have lesser vascular density or vascular appearance upon angiography, and they may show a low degree of EPR effect or low vascular density as discussed earlier. People who do not accept the existence of the EPR effect have noted this point as heterogeneity of EPR effect. A hypovascular nature (or heterogeneity of EPR effect) indicates the presence of insufficient angiogenesis for tumor growth. However, blood vessels always occur wherever tissues grow; that is, no tissue lacks blood vessels (with an exception of cartridge). In the window chamber model of solid tumors in rats, one can observe very irregular blood vessels [89], and blood flow may be seen only irregulary, once every, eg. 17 or 21 min unless blood pressure is raised. Hori and colleagues [89,90] also found that blood flow direction may change suddenly. Colloidal osmotic pressure in these solid tumors is believed to become very high and hence suppress penetration of drugs into tumor tissue. One argument proposes that mechanical tissue pressure is generated in an artificial chamber, where tumor cells have a doubling time of 24–48 h, and thus the tissue mass will fill up the chamber and compress the chamber space after a certain time interval, which would thereby impede vascular blood return or normal vascular physiology.

In addition, most tumor cells have adapted hypoxic or anaerobic metabolism for energy production (known as the Warburg effect). Under such conditions, hypoxia-inducible factor, HIF-1α, is known to be activated

and to lead to generation of VEGF. Because the EPR effect is the result of vascular leakage of macromolecules from the luminal side of blood vessels to the tumor interstitium to support nutrients and oxygen supply, I initiated further investigations to enhance the EPR effect more by two practical methods for drug delivery to tumors.

10.A. By Elevating Blood Pressure

The first method involved artificially inducing the hypertensive state (e.g., from 110 to 150 mmHg) with a slow intravenous infusion of angiotensin-II (AT-II). This pathophysiology of tumor vasculature was first described by Prof. Maro Suzuki [90]. Drug in circulation would thus be more effectively pushed into tumor tissue because of the increased vascular pressure would open up only in the tumor vasculature due to incomplete vascular architecture such as lack of smooth muscle layer surrounding the blood vessels (see Figure 7 B(2)).

Regardless of apparently low vascular density tumor, much more blood vasculature can become visible (Figure 11, A→B) when higher blood pressure is generated by slow infusion of angiotensin II (from 100-120 to 150-160 mmHg), by using the angiographic technique (Figure 7 B(2), Figure 11 A, B).

In consistent with this notion, enhanced vascular flow, and hence the drug delivery of SMANCS/Lipiodol to actual human tumor with apparently low vascular tumor (which exhibiting less EPR effect) could be augmented (Figure 11 C,E,G → D,E,F and Figure 12, Case 1-7; Figure 13).

In an AT-II-generated hypertensive state, the endothelial cell-cell gap junctions in normal tissues would become tight, so fewer drugs would leak out of vessels in normal tissues in contrast to tumor vessels which would be opened up (Figure 7B). These experiments were carried out in 1991 by my then-student Chang Li (67), from China, who later received his PhD and MD degrees from Harvard and is now CEO and CSO of Boston Biomedical Inc. and was on the faculty at Harvard Medical School and CEO of ArQule. Clinical evaluation of this method was more recently conducted by my colleagues Dr. Akinori Nagamitsu and Dr. Khaled Greish in our Hakuaikai hospital in Kumamoto, with highly encouraging results that were recently published [66]. Some clinical results of advanced difficult-to-treat tumors are shown in Figure 12 and 13, who were treated under the angiotensin II induced higher blood pressure [66].

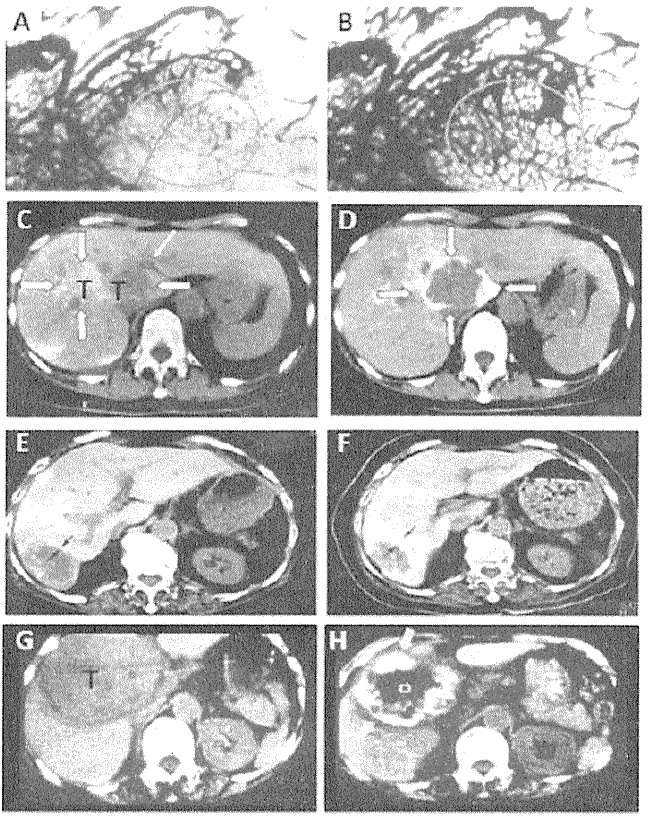

Figure 11. Augmentation of the EPR effect by angiotensin II (AT) induced high blood pressure. Top A/B. A window chamber model of an experimental rat tumor model. Blood vessels are only weekly seen under normotensive state (circled area by pink color) in (A), but the blood vessels became denser as noted in B when the systolic blood pressure of 90 mm was elevated to 160 mmHg (B) (see pink circled area). (adapted from ref. *90,91*). The following examples in Figure 11-13 [66] are results of arterial infusion of SMANCS/Lipiodol® of normotensive blood pressure (90-120 mmHg) (C,E,G) and AT II infused condition (to about 150-160 mmHg)(D,E,F). Both C/D and E/F are colon cancer → liver metastasis; G/H is a case with massive gallbladder cancer metastasized to the liver. In all these (C,E,G) cases are difficult-to-treat cases. Under the angiotensin II induced hypertensive state, CT scan images clearly showed significantly enhanced drug delivery and therapeutic effect [66].

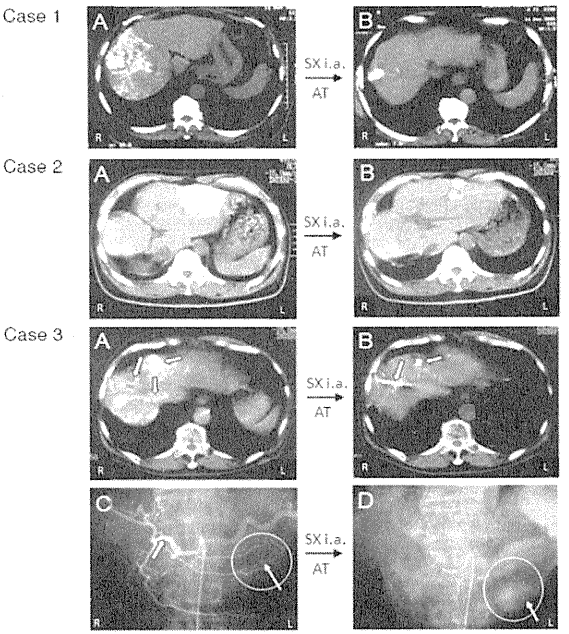

Figure 12. Therapeutic effect of SMANCS (SX)/Lipiodol (LP) on hepatocellular carcinoma (HCC) given via the hepatic artery under the angiotensin II (AT) induced high blood pressure. (Case 1): Response of recurrent HCC to SMANCS/LP under AT-induced hypertension (AT/hyper) 3 years after resection. Reduction in the size of the major tumor in 1 month after only one treatment was remarkable (A vs. B). (Case 2): A massive HCC treated with SMANCS/LP (3 ml) under AT/hyper. A considerable tumor size reduction in 1 month was obtained (A vs. B). (Case 3): Another case of recurrent HCC 2.5 years after resection. The patient received i.a. SMANCS/LP under AT/hyper. The arterio-portal shunts are seen in (A and B). In (C) (angiogram), unique feeding artery branching off from the right proper hepatic artery (arrow) extending to left lower part (encircled tumor) is seen in (D). (B) CT after 6 months, which showed a definite reduction in tumor volume from (A). Enhanced tumor stain (drug delivery) in (D), lower left circled, is seen in the angiogram under AT/hyper administration.

Application of this SMANCS under elevated blood pressure was carried out not only for the primary liver cancer (Figure 12) but also many tumors at advanced stage of metastatic liver cancers from colon cancer, gallbladder cancer (Fig. 11, C, E, G), from stomach cancer, pancreatic cancer, ovarian cancer (Fig. 13, case 4-6), and massive renal cell carcinoma (Fig. 14 (1), (2)) [66]. All cases showed remarkable response [66].

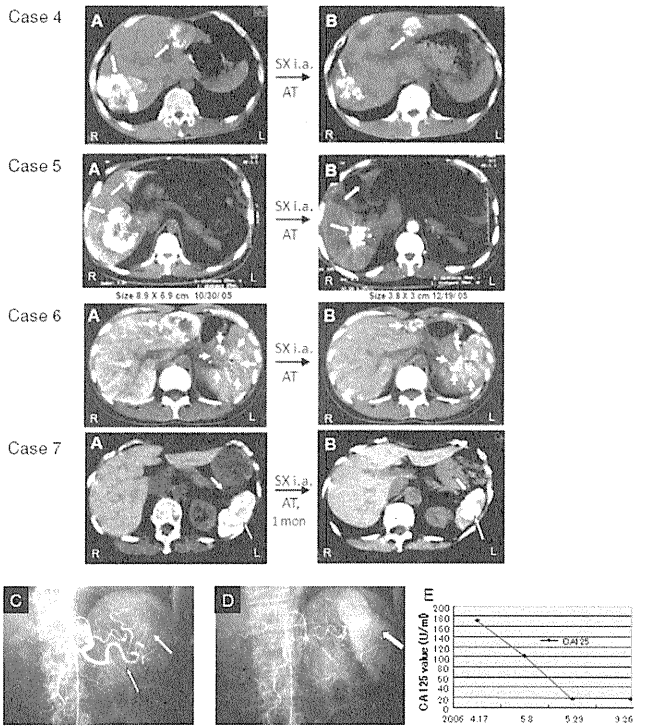

Figure 13. Responses of various metastatic liver cancers to SMANCS/Lipiodol under AT/hyper. (Case 4) A case of stomach cancer metastatic to the liver after resection 3 years earlier. Both posterior and anterior metastatic liver tumors showed marked reductions in tumor volume found 8 months after i.a. SMANCS/Lipiodol infusion under AT/hyper. (Case 5) A massive metastatic liver cancer originating from stomach cancer regressed considerably in 50 days. The B-type staining (peripheral ring shape), usually seen in metastatic tumors by CT, indicates greater drug uptake under AT/hyper (both case 4A and 5A) than under normotensive conditions (data not shown). (Case 6) Massive metastatic liver cancer originating from pancreatic cancer. (A) CT of both the metastatic mass in the liver [center front in (A)] and the primary pancreatic cancer (left middle) at the time of the first infusion of SMANCS/Lipiodol. A large metastatic tumor mass in the frontal area regressed considerably (B) in 5 months after one injection. The primary pancreatic cancer taken up the SMANCS/Lipiodol also (Case 6, Fig. B). (Case 7) Ovarian cancer, of which original tumor had been removed surgically 1.5 yr before. However, it metastasized to the spleen and SMANCS/Lipiodol was infused under AT/hyper (C/D). After 1 month, it showed a good response (B), which corresponded to the decrease of CA125 tumor marker after SMANCS/ Lipiodol (E). Angiography under AT/hyper shows the splenic artery and tumor in the spleen (C and D, white arrow) that is visible in the initial CT scan (A) and significant reduction after 1 month (B and D, heavy tumor stain). Clear radiodense area (arrow in B and D) indicates high uptake of SMANCS/Lipiodol.

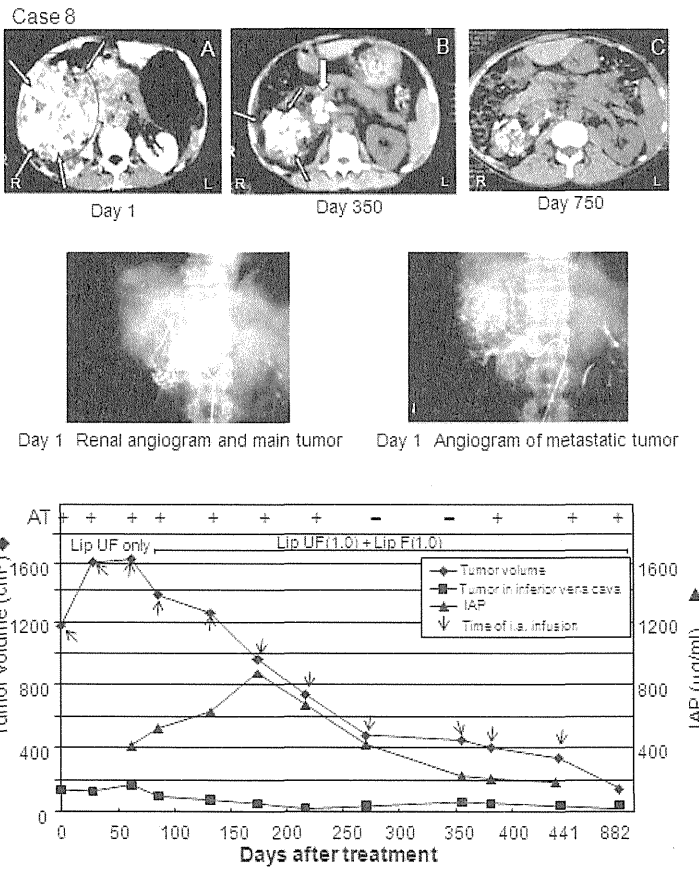

Figure 14. Renal cell carcinoma treated with SX/LP under AT/hyper. (1) (A–C) CT scans of Case 8 on Day 1, Day 350 and Day 750 over time. An initial renal angiogram showing major renal tumor in (D) and also metastatic tumor in the inferior vena cava in (E) seen under angiography, both on Day 1. (2) Time course of tumor volume (closed diamond), tumor marker (immunosuppressive acidic glycoprotein, IAP) values (closed triangle) and estimated volume of metastatic tumor nodule in the inferior vena cava (closed square). The times of i.a. infusions of SMANCS/LP are indicated by short arrows and use of AT/hyper on the top by (+).

Because of my pioneering work on polymeric drugs, particularly SMANCS, and discovery of the EPR effect of macromolecular drugs in solid tumor, I received a Lifetime Achievement Award by *Journal of Drug Targeting*, Informa UK Ltd. (publisher) at the Royal Pharmaceutical Society,

in Manchester, in 2007. Also, the *Journal of Drug Targeting* published a special issue to honor my award in 2007. The previous winners of this Award are R. L. Juliano (USA,2004), A. Florence (UK, 2005) and H. Ringsdorf (Germany, 2006).

10.B. By Using NO Releasing Agents

The second method to enhance EPR effect (or to overcome the heterogeneity of EPR effect) is to utilize NO. We recently found the extremely interesting fact that drug delivery is enhanced by externally applied nitroglycerin (*92,93*). Nitroglycerin has been used for more than 100 years to treat myocardial infarction and angina pectoris. Infarcted myocardial tissue becomes hypoxic, similar to many tumor tissues, so that both tissue oxygen tension (pO_2) and pH values are low if not all cases. Nitroglycerin is readily absorbed from the dermal surface into the circulation, and nitrite ion (NO_2^-) is generated from nitroglycerin (via denitrase) in the hypoxic infarcted tissue. NO_2^- is then converted to NO by nitrite reductase (see Figure 15, below). As described earlier, NO is one of the major vascular permeability factors and facilitates the EPR effect, primarily in tumor tissue. This NO_2^- release thus occurs very similarly or in the same manner, in both infarcted cardiac tissue and tumor tissue. The result is indeed enhanced drug delivery to tumors and an improved therapeutic effect. Takahiro Seki (our postdoctoral fellow), Jun Fang, and I recently reported this finding [92, 93].

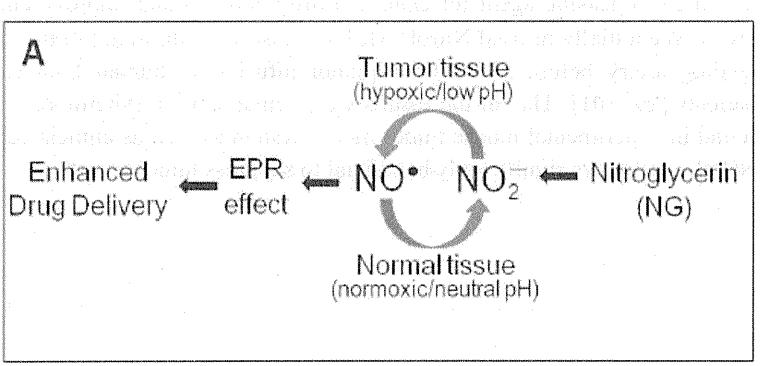

Figure 15. (Continued).

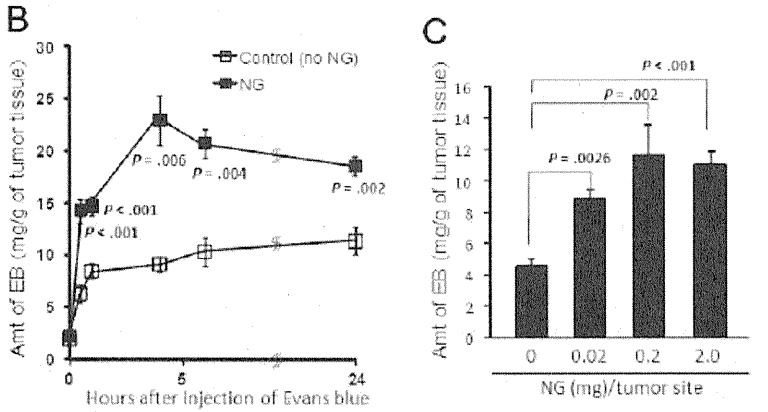

Figure 15. A. Mechanism of nitroglycerin induced augumentation of EPR effect and conversion of nitroglycerin to NO_2^-, then to NO in tumor tissue. B. Increased tumor delivery of Evans blue albumin (macromolecules of 67KDa) in time dependent manner with nitroglycerin (NG) application on the skin. C. Dose dependency of NG in enhancement of EPR effect [93].

Application of this nitrite releasing agent in the clinical setting has been undertaken by Yasuda et al in conventional cancer chemotherapy [94-96]. Their working hypothesis was based on earlier funding that nitroglycerin improved pO_2 of tumor tissue thereby affecting the signaling cascade of cancer tissue including down regulation of vascular endothelial growth factor (VEGF), hypoxia inducible factor and MAP-kinase, etc [97,98]. In different context, since we know NO facilitates EPR-effect, we examined the use of NO releasing agent for enhanced drug delivery and improve clinical effect. We initially infused Nitrol® (ISDN; isosorbitol dinitrate) to the tumor feeding artery before SMANCS/Lipiodol infusion in human lung cancer patients [99, 101]. The all the results were remarkable. Furthermore, it was found in experimental mouse tumor model system as well as clinical setting, NO alone appears significantly beneficial to suppress tumor growth.

Chapter 11

SMA AS A VERSATILE MICELLE-FORMING AGENT

Dr. Khaled Greish from Egypt joined my department as a graduate student in 2003. He had clinical experience (with a Master's degree in medicine) and a keen interest in new areas of cancer chemotherapy. I assigned him to investigate the possibility of new micelle formation with various anticancer agents using SMA, because of my earlier experience, I knew that SMA formed micelles. The first issue was which drugs to encapsulate in the micelles, and then, how to make the micelles, and what would be the characteristics of the SMA micelles. For a model, we chose the anthracyclines at first: doxorubicin, aclarubicin, taxol, cisplatin and then pirarubicin (tetrahydropyranyl-doxorubicin, or THP). They all formed useful micelles or complex, and particularly with THP, the micelle exhibited slower drug release and more stable than doxorubicin micelles, and indeed, SMA-THP micelles showed excellent in vivo antitumor activity [102-106].

A few years before our investigations of SMA micelles, PEGylated ZnPP (PEG-ZnPP) had been shown to have excellent antitumor properties in vivo; our postdoctoral fellow from India, Dr. S. K. Sahoo [107], and Jun Fang (who is an associate professor, now at Sojo Univ.) in our laboratory described the in vivo evaluation [108]. ZnPP is almost insoluble in water, but PEG-ZnPP and SMA-ZnPP micelles became water-soluble; these ZnPP derivatives exhibit inhibitory effect to heme oxygenase-1 (HO-1). HO-1 is also called heat shock protein (Hsp) 32 and knows as survival factor of cancer cell; HO-1 is highly upregulated in most cancer cells. In these cells, HO-1 generates biliverdin and CO (carbon monoxide) and iron from heme, and biliverdin is then oxidized to bilirubin. We realized that because bilirubin is a potent antioxidant, it confers antioxidative power to tumor cells

to protect them against endogenous oxidants (or ROS, reactive oxygen species). ROS may be generated by leukocytes and anticancer agents. Administration of PEG-ZnPP, to suppress bilirubin production in vivo, would thus make target tumor more vulnerable to ROS, thus useful as anticancer agent [107-111].

More recently such encapsulation of zinc protoporphyrin (ZnPP) into SMA- micelles after the work of PEG-ZnPP was found intriguing [104,111-113]. Details such as drug release, stability, tumor targeting via the EPR effect, both therapeutic effect and toxicity in vivo were clarified by J. Fang, K. Greish, and A. K. Iyer (another graduate student from India). All the micellar drugs were better than the parental free drugs in pharmacokinetics, therapeutic effect and toxicity wise. The SMA-pirarubicin micelle (Figure 16) was the best among them: it had a longer (about 200 times) plasma circulation time and a higher (more than 20 times) tumor accumulation than the parent pirarubicin. In tumor-bearing mice, even at one-fifth of the maximal tolerable dose (i.e., a very low dose), SMA-pirarubicin micelles produced 100% survival at more than 200 days without detectable toxicity in S-180 tumor bearing mouse model together with pathological data using the vascular cast and scanning electron microgram, the paper was published recently in Cancer Science as highlighted paper [106].

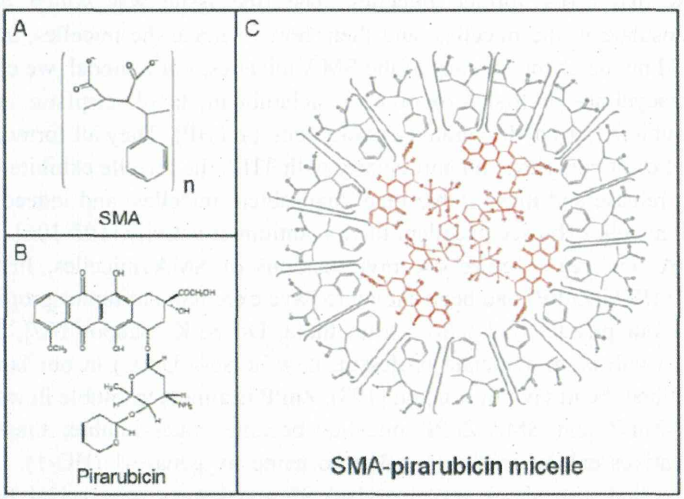

Figure 16. Representation of the chemical structures. (A) Styrene-maleic acid copolymer (SMA). (B) Anticancer agent pirarubicin, THP. (C) Putative structure of SMA-pirarubicin (THP) micelles. Brackets indicate copolymer of SMA (n=10-15).

Our report on the anticancer effects of the HO-1 inhibitor PEG-ZnPP drew the attention of many researchers throughout the world. One of them, Professor Peter Valent (Division of Hematology and Hemostaseology at the Medical University of Vienna), asked me to supply SMA-ZnPP and PEG-ZnPP. Also, from the Humboldt University of Berlin, Professor Beate Raeder (a laser biophysicist) asked me to collaborate. Both collaborations worked very well. Professor Valent's group published a paper on SMA-ZnPP and PEG-ZnPP that showed inhibition of HO-1, and interestingly downregulation of an oncogene *bcl/abl* in chronic myelogenous leukemia cells and other lymphocytic leukemia cells. They showed that SMA-ZnPP micelle was also effective against imatinib-resistant chronic myelogenous leukemia cells. Thus, HO-1 is an ideal molecular therapeutic target for inhibition without toxicity [112-114].

Furthermore, SMA can encapsulate various fluorescent dyes such as fluorescein, rose bengal, and indocyanine green (as recently demonstrated by G. Bharate, a PhD student) as well as chlorophyll studied by Hideaki Nakamura, our junior faculty member) (unpublished data), and we expect future development along this line.

Another important aspect of SMA-micelle we found is that SMA micelles undergo disintegration, and then liberate the drugs from the micelles upon endocytotic intracellular uptake, similar to uncoating of virus capsid/virion upon viral infection; in that process viral genome is liberated free in the cytoplan. This notion was demonstrated experimentally by Hideaki Nakamura recently, which I believe will draw attention of the scientist working on liposome and micelles [115].

We have reported many fluorescent dyes becomes non-fluorescent in SMA micelles due to compact packing in the micelles [102-104] that result in energy transfer and fail to emit fluorescence. Further, the quenched fluorescence can be regenerated by micelle destabilizing agents such as sodium dodecyl sulfate (SDS), ethanol, and lecithin [102, 103, 115]. In the liposome research, it is known that the control of liberation of the encapsulated drugs is one of the most important key point, and people use ultrasonic, heating, or shifting pH, or using environment sensitive polymers, etc to control release velocity. The present finding of drug release upon intracellular uptake is thus so important and ideal. In this setting, amphiphilic component like lecithin in the cell membrane will serve endogenous drug releasing component, therefore SMA micelles, thus appear more ideal than other types of liposomes or micelles.

Chapter 12

PROTEIN DRUGS, THEN AND NOW

During development of proteinaceous pharmaceuticals in medicine, the possible antigenic nature of many proteins has been the first concern, and the labile nature of proteins has been the second. We showed earlier that one could overcome these problems via polymer conjugation, including modification with SMA, which nullified the immunogenicity of SMANCS and prolonged its in vivo half-life [29-31,116-119]. During this period, PEGylation was becoming a standard procedure used to reduce the immunogenicity of proteins. Before NCS was approved in 1971 as a therapeutic agent for treatment of leukemia and cancers of the gastrointestinal tract, pharmaceutical community had very little experience with or knowledge about protein drugs in technical and scientific issues, such as pharmacokinetics, tissue distribution, AUC (area under the concentration-time curve), and inactivation of protein drug.. The protein nature of NCS meant that proteolytic degradation would occur during sample preparation or processing, as well as on administration. Thus, NCS activity would drop rapidly if no protease inhibitor were used [29, 30,116-119]. My experience in Professor Feeney's laboratory helped me clarify these points. However, protein drugs were so new at that time that general acceptance in clinic was not so easy to achieve. NCS has, as mentioned earlier, extremely high activity at or less than the nanomolar range, and is thus very toxic unless the dosage is carefully controlled. Furthermore, its urinary excretion is extremely fast, with its molecular size of about 12 kDa, and its in vivo proteolytic degradation is also very rapid [26,116]. It is thus essential for good therapeutic effect to maintain a meticulously controlled plasma concentration without over shooting the drug level to avoid adverse effect. We published these pharmacokinetic data for the theoretical calculation of

infusing velocity for brain tumor via the intracarotid-arterial, and intravenous infusions velocity for leukemia, in which infusion of NCS was determined by considering the rates of elimination (urinary excretion) and inactivation in the blood, and the IC_{50} etc [27,28,121]. However, precise fine-tuning of dosing velocity was not popular among clinicians at that time.

On the occasion of 30 years of the discovery of prototype proteinaceous antitumor agent NCS, we organized a symposium on NCS in Kumamoto in 1994. NCS had become a leading prototype of protein antitumor agents that contains unique endyene chromophore as active moiety that generates reactive oxygen species. Namely, proteinaceous antitumor agents included actinoxanthin in Moscow (Prof. Khoklov), macromomycin in Tokyo (Prof. Umezawa), and lymphomycin and largomycin and others in Sendai (Prof. Ishida). The book on NCS describes the general characteristic of neocarzinostatin, the amino acid sequence and chemical structure of the protein portion and the chromophore, two-dimensional NMR study, X-ray crystallography, the mode of action at the molecular level, and the immunopotentiating effect. Clinical effects and side effects are also discussed, as well as development of the polymer conjugation for the development of anticancer polymer drug SMANCS and its clinical application [121].

Our second macromolecular drug, SMANCS, which the Japanese Government approved for its use in 1993, was launched for marketing in 1994 by Yamanouchi Pharmaceutical Company, Tokyo, but its acceptance was also not so straightforward. One issue was that its approved route of administration was intra-arterial, into the hepatic artery, for treatment of hepatocellular carcinoma (primary liver cancer). A second issue concerned the market size and sales volume. The financial incentive for any drug with a sales volume of less than $10 million or even $100 million dollars (US) per year is not attractive to most pharmaceutical companies, so that not much interest existed for expanding market, or extension for approved therapeutic use for other types of cancers. In addition to these facts, SMANCS required mixing with Lipiodol under ultrasonic at the theater of angiographic procedure as it is supplied separately from Lipiodol, and this made it too awkward step in the busy clinic. Thus, its clinical development other than hepatoma received no support from the manufacture.

Today, a few decades later, protein drugs, many of which are PEGylated proteins, are attracting more attention than ever. According to *Chemical and Engineering News* of July 20, 2009, 4 of the top-selling 15 U.S. pharmaceutical products in 2008 were protein drugs. However, for the category of anticancer drugs of so called molecular target drugs, questions

about cost/benefit issues prevail. Namely, many of the molecular target drugs do not satisfy most of the patients. For instance, Tito Fojo and Christine Grady [122], and others [123,124] have expressed concerns about this issue, and The Lancet carried Editorial comment and other article on this matter.

However, I can see great potential for development of anticancer drugs based on the EPR effect, which could target all tumors more selectively and universally. Furthermore, we have now found ways to augment the EPR effect 2- to 3-fold by simple clinical manipulations, as discussed above. Therefore, macromolecular drugs may be more effective than low-molecular-weight drugs, because these EPR effect-based drugs utilize more universal tumor targeting and ubiquitous characteristics of solid tumors as well as inflammatory tissues.

Another issue is related to the system of anticancer drug development, about which one must think more carefully and wisely. For example, the drug-screening mouse models do not truly mimic highly mutated or genetically diversified human solid tumors, because mouse model tumor cell lines have features of primarily one clone and no host reactions (meaning no inflammation → no free radicals → no mutation → no genetic divergence) [125, 126]. I believe that the design of synthetic polymeric drugs will be more economical and effective, which have much better potential of cost/benefit performance than the available contemporary protein drugs such as monoclonal antibodies, which are extremely expensive and selective to one specific target molecule (epitopic) of tumor cells. Thus, these macromolecular drugs will eventually be adopted for use in oncology clinics. In fact, many polymeric drugs are now in phase I and II trials in the United States, Japan, and Europe, and I am excited to see their clinical successes.

Chapter 13

ORGANIZING VARIOUS ACADEMIC MEETINGS

My research activities, in addition to involving bacterial proteases and polymeric drugs, focused on clarifying the roles of endogenous free radicals (e.g., $O_2^{\bullet -}$ and $NO^{\bullet}$) in infection and cancer in the late 1980s to 2004 before the mandatory retirement from Kumamoto University. Incidentally, I organized two international meetings on NO—one in 1997 in Kyoto, Japan, with Professor Noboru Toda (Shiga University) and Professor Salvador Moncada (The Wolfson Institute for Biomedical Research at University College London), and another one, The 3rd International Conference on the Biology, Chemistry, and Therapeutic Applications of Nitric Oxide in 2004 in Nara, Japan, with Professor Mitsuhiro Yokoyama (Kobe University) and Professor Naoyuki Taniguchi (Osaka University). The International Nitric Oxide Society, of which I was president for 2 years, published a special issue of the journal *Nitric Oxide: Biology and Chemistry* to honor my retirement in 2005. This became the third one after *Biological Chemistry* (German Biochemical Society) and *Journal of Drug Targeting* from UK as described. I am also helped to organize four other meetings in Kumamoto, Japan, in the past 7 years: 9th Meeting of the Society of Cancer Prevention of Japan in June, 2002; the 3rd Japan NO Meeting in May, 2003; the 76th Annual Meeting of the Japanese Society for Bacteriology in April, 2003; and the 23rd Meeting of the Japan Society of Drug Delivery System, in June, 2007. Through all these academic activities, I made so many friends nationally and internationally and was a great reward in my scientific carrier.

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NOTE

On November 3, 2010, I was awarded prestigious 70th Nishi-nippon Culture Award from Nishi-nippon Shimbun (News agency in Fukuoka, Japan). On June 27, 2011, The Japan Society of Drug Delivery System awarded me its highest award, The 11th Nagai Award. More recently, on October 5, 2011, Japanese Cancer Association awarded me The Tomozo Yoshida Award of 2011, the most prestigious award of the society for which I am most honored. Each of these award is given to one person a year.

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