

BPB Reports

Regular Article

A Safety Evaluation Study in Mice Revealed that Albumin Dimer is Safe for Medical and Pharmaceutical Applications

Mai Hashimoto,^{#,a} Victor Tuan Giam Chuang,^{#,b} Yu Ishima,^c Mayumi Ikeda,^c Toru Maruyama,^d Keishi Yamasaki,^{a,e} Kazuaki Taguchi,^{*,f} and Masaki Otagiri^{*,a,e}

^aFaculty of Pharmaceutical Sciences, Sojo University, 4-22-1 Ikeda, Nishi-ku, Kumamoto 860-0082, Japan; ^bSchool of Pharmacy, Monash University Malaysia, Malaysia Campus, Jalan Lagoon Selatan, Bander Sunway 47500, Subang Jaya, Selangor, Malaysia; ^cDepartment of Pharmacokinetics and Biopharmaceutics, Institute of Biomedical Sciences, Tokushima University, 1-78-1 Shomachi, Tokushima 770-8505, Japan; ^dGraduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Chuo-ku, Kumamoto 862-0973, Japan; ^eDDS Research Institute, Sojo University, 4-22-1 Ikeda, Nishi-ku, Kumamoto 860-0062, Japan; ^fFaculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan

Received March 24, 2020; Accepted April 6, 2020

Human serum albumin (HSA) dimer, where two molecules of HSA are genetically fused with a linker of 10 amino acid, has superior blood retention property, compared with HSA monomer. Moreover, HSA dimer derivative, s-nitrosated HSA dimer, functions as an enhanced permeability and retention effects enhancer. HSA dimer has gained considerable attention as drug delivery system carrier based on its prominent function. However, for the HSA dimer to be used clinically, the safety profile of the HSA dimer is required in order to exclude any potential toxicity or unwanted effects. Thus, the present study was undertaken to investigate the occurrence of tissue damage and serologic changes due to repeated administration of HSA dimer (66.5 mg/kg) to mice every 3 d for 56 d, as part of a basic consideration on safety evaluation. The evaluation on survival, behavior and body weight indicate that HSA dimer has no effect on physical growth and physiological functions. Hematological tests suggest that HSA dimer has no direct influence on hemocytes, such as hemolysis and platelet aggregation. Moreover, plasma clinical chemistry and histological examinations indicate that the HSA dimer has no deleterious effect on liver and renal functions. The results obtained here indicate HSA dimer is safe and should be useful for medical and pharmaceutical applications.

Key words albumin, dimer, toxicity tests, safety evaluation, medical and pharmaceutical application

INTRODUCTION

Human serum albumin (HSA) is the most abundant protein in plasma and is widely used in clinical practice as human blood product for maintaining oncotic pressure.^{1,2} The half-life of HSA in human blood is about 20 d, which is longer than other plasma proteins. Taking advantage of this long blood retention property of HSA, attempts to develop its application in medicines, particularly as drug delivery system (DDS) carrier, has been more active than ever.^{2,3} In fact, DDS preparations utilizing HSA, such as glucagon-like peptide-1 (GLP-1) HSA fusion (TanzeumTM) and nanoparticle preparations of anticancer drug Paclitaxel and HSA (Abraxane[®]), are increasing.^{4,5}

On the other hand, various HSA mutants, dimers, and the like have been created with the progress of genetic engineering, and research on their applications for medical use has been actively conducted. For example, we have produced and examined the kinetic properties of a HSA dimer, and found its blood half-life was significantly longer than that of the monomer.⁶

Further modification of the dimer, such as S-nitrosated HSA dimer (SNO-HSA-dimer), imparted new function to the dimer as an EPR (Enhanced Permeability and Retention) enhancer.⁷⁻⁹

In order to materialize the potential clinical use of HSA dimer, further work is essential to establish the safety profile of the HSA dimer. Therefore, in this work, as part of a basic study on safety evaluation, changes in body weight, tissue damage (liver, kidney, lung) and serologic changes due to repeated administration were examined. The results obtained in this study provide fundamental safety information for developing HSA dimer as a carrier of nitric oxide for EPR enhancer.

MATERIALS AND METHODS

Preparation of HSA Monomer and Dimer HSA monomer was purchased from Sigma-Aldrich (St. Louis, MO). HSA dimer were synthesized using the yeast *Pichia pastoris* (strain GS115) as previously described.^{6,7}

Animals Male BALB/cCrSlc mice (5 weeks old, 18–23 g)

*To whom correspondence should be addressed. e-mail: taguchi-kz@pha.keio.ac.jp
e-mail: otagirim@ph.sojo-u.ac.jp

[#] These authors contributed equally to the work.

were purchased from Japan SLC, Inc (Sizuoka, Japan). The mice were maintained in a conventional room in which stable conditions of temperature and humidity with a standardized light/dark cycle. The animals were treated in accordance with the National Institutes of Health guidelines. All animal experiments were reviewed and approved by the Animal Care and Use committee of Sojo University.

Subacute Toxicity Experiments Saline, or HSA monomer and dimer dissolved in saline, were intraperitoneally administered to mice every 3 d (HSA monomer: 133 mg/kg, HSA dimer: 66.5 mg/kg). The dosage of HSA dimer is similar to those used in previous studies where SNO-HSA-dimer functioned as an EPR enhancer.⁷⁻⁹⁾ Before administering each test sample, the weight of mice and their appearance and behavior were recorded. At 28 and 56 d after the first administration, the mice were randomly selected and sacrificed for collecting blood and organs (lungs, liver and kidneys). After analyzing hematological parameters using an animal blood cell counter (MEK-6458; NIHON KOHDEN Corp., Tokyo, Japan), the blood samples were centrifuged (3,000 rpm, 10 min) to obtain plasma for analysis of the following clinical chemistries: albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea nitrogen (BUN), creatine kinase (CK) and creatinine. All plasma samples were stored at -80°C prior to analysis by a commercial clinical testing laboratory (Oriental Yeast Co., Tokyo, Japan). Paraffin embedded sections of lungs, liver and kidneys were stained with hematoxylin and eosin (HE) and subsequently observed histology using a BZ-X710 microscope (Keyence, Osaka, Japan).

Statistical Analysis All data are expressed as the mean \pm SD. Statistical analyses for multiple comparisons in the study were determined by the analysis of variance (two-way ANOVA) followed by the Bonferroni analysis. A probability value of $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Survival, Behavior and Body Weight All mice that have been repeatedly administered either HSA monomer or dimer every 3 d survived up to 56 d until manually sacrificed for data collection. In addition, there was no abnormal behavior, no reduction in appetite and no changes in appearance dur-

ing observed period. Body weight of mice in both HSA monomer and dimer groups increased in a comparable manner as the saline group throughout the period of observation (Fig. 1). These results indicate that HSA dimer has no deleterious effect on physical growth and physiological functions.

Hematological Tests The number of white blood cell (WBC) was slightly increased on 56 d in HSA monomer and dimer groups, but there was no significant difference among groups (Fig. 2A). No abnormal change of number of red blood cell (RBC) and platelet at 28 and 56 d in HSA dimer group (Fig. 2B and 2C). There were also no changes or significant difference of RBCs, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration (data not shown). These results suggest that HSA dimer has no direct influence on hemocytes, such as hemolysis and platelet aggregation, and has no effect on the production of hemocytes.

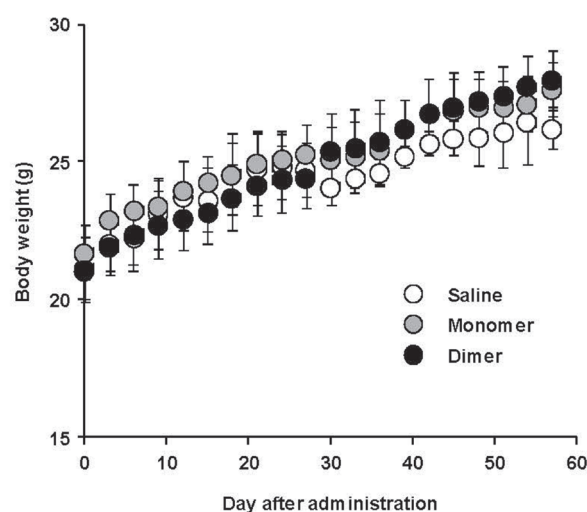


Fig. 1. Changes in Body Weight for 56 d After the Start of Saline (Open Circles), HSA Monomer (Gray Circles) and HSA Dimer (Closed Circles) Administration to Healthy Mice

The number of mice (n) that have been administered saline, HSA monomer and HSA dimer group was $n=6$, $n=15$ and $n=14$, respectively. At 28 d after the start of test samples administration, the mice in saline ($n=3$), HSA monomer ($n=7$) and HSA dimer ($n=7$) group were planned sacrifice for collection of blood and organ samples. Each value represents the mean \pm S.D.

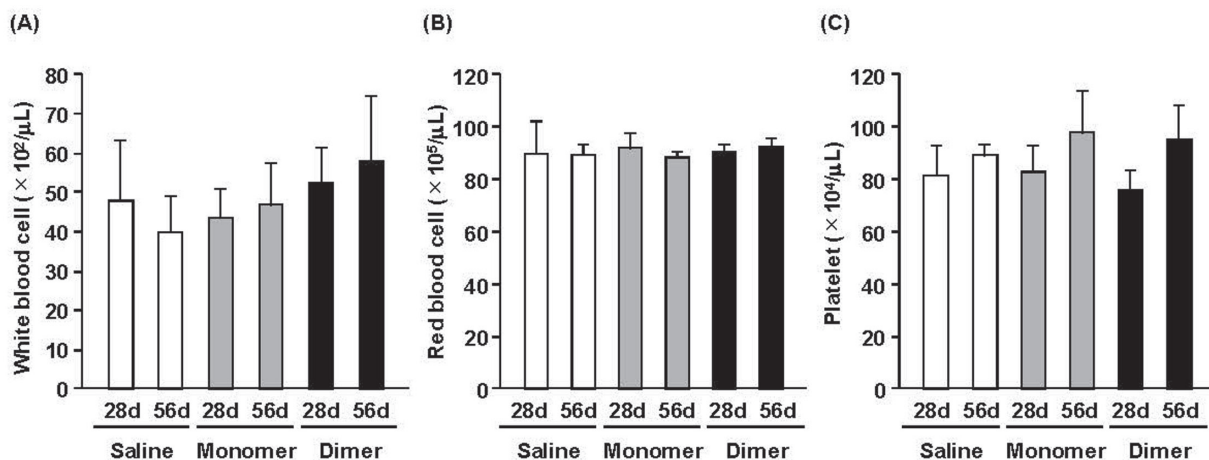


Fig. 2. The Numbers of (A) White Blood Cells, (B) Red Blood Cells and (C) Platelets at 28 and 56 d After the Start of Saline (Open Columns), HSA Monomer (Gray Columns) and HSA Dimer (Closed Columns) Administration to Healthy Mice

The number of mice (n) in saline (28 d), saline (56 d), HSA monomer (28 d), HSA monomer (56 d), HSA dimer (28 d) and HSA dimer (56 d) group was $n=3$, $n=3$, $n=7$, $n=8$, $n=7$ and $n=7$, respectively. Each column represents the mean \pm S.D.

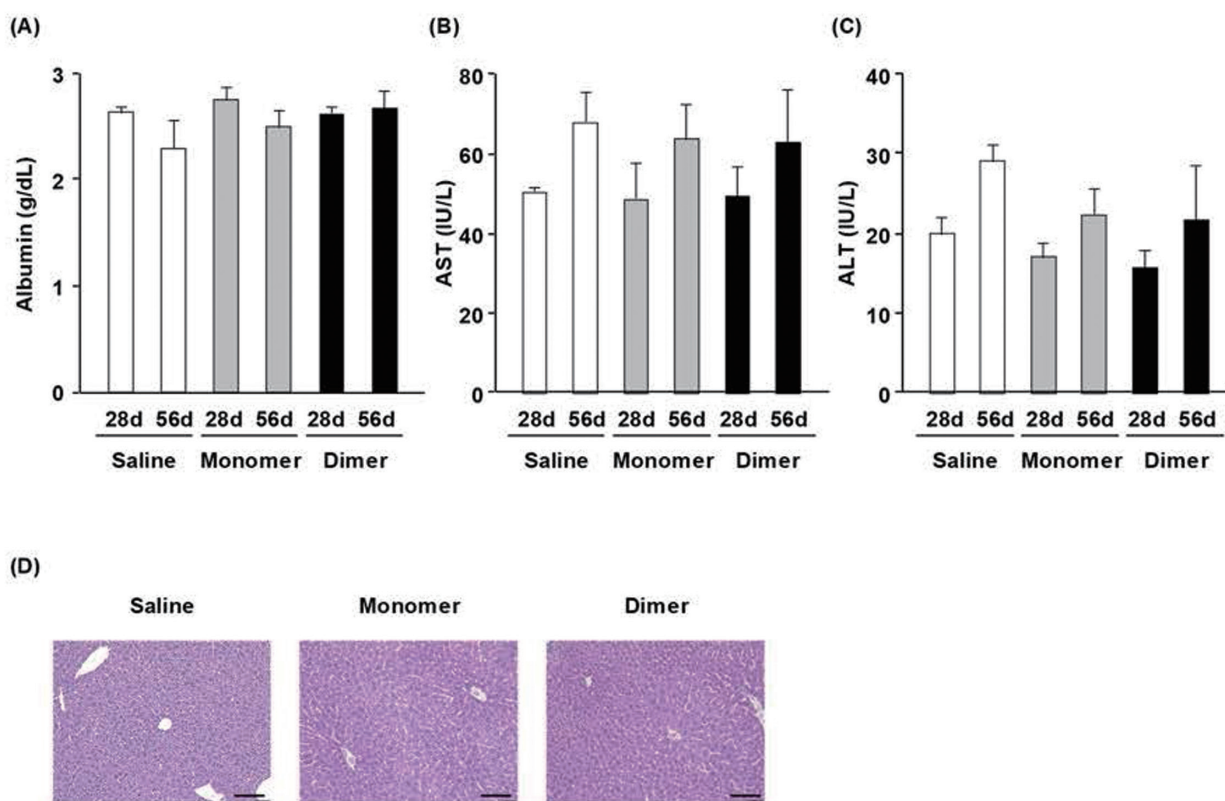


Fig. 3. Plasma Clinical Chemistries ((A) Albumin, (B) AST and (C) ALT) Representing Hepatic Function at 28 and 56 d After the Start of Saline (Open Columns), HSA Monomer (Gray Columns) and HSA Dimer (Closed Columns) Administration to Healthy Mice. (D) Light Micrographs of Livers Stained with H&E

The number of mice (n) in saline (28 d), saline (56 d), HSA monomer (28 d), HSA monomer (56 d), HSA dimer (28 d) and HSA dimer (56 d) group was n=3, n=3, n=7, n=8, n=7 and n=7, respectively. Each column represents the mean \pm S.D. Scar bar represents 100 μ m.

In addition to the influence of HSA dimer on hemocytes, the immunogenicity of HSA dimer is also a concern. So far, there was no incidence of anaphylaxis or immunological responses reported after single and multiple administration of both chemical and genetic HSA dimers.^{10–12} Thus, no specific anti-body against HSA dimer was produced. However, immunological responses of HSA dimer will need to be monitored to accumulate safety evidence.

Plasma Clinical Chemistry and Histology for Evaluation of Hepatic Function The liver is the main organ responsible for the distribution and metabolism of both HSA monomer and dimer after exogenous administration. Both HSA preparations are likely to be captured by Kupffer cells.^{6,13} Therefore, HSA dimer administration might influence the hepatic functions. In this study, the levels of albumin, AST and ALT were examined as a marker of liver function at 28 and 56 d after the start of administration of each test sample. No significant difference in albumin and AST at 56 d were found among test samples (Fig. 3A and 3B). However, the levels of ALT at 56 d after administration were slightly decreased in HSA monomer and dimer groups, but these reductions were within normal range (Fig. 3C). No changes in the histopathological examination were found among groups (Fig. 3D). These results indicate that HSA dimer has no deleterious effect on liver function.

Plasma Clinical Chemistry and Histology for Evaluation of Renal Function Both HSA monomer and dimer are also distributed to kidneys after exogenous administration.^{6,13} There are various kinds of receptor, such as neonatal Fc receptor, cubulin and megalin, which involved in regulating the pharmacokinetic of albumin.^{14,15} Hence, the effects of

HSA dimer on renal function were also evaluated by the measurement of BUN and creatinine levels in plasma. As a result, the level of BUN was slightly decreased at 56 d in the HSA monomer and dimer groups, but these changes were still within the normal range (Fig. 4A). Interestingly, the values of creatinine were found to be increased in all groups (Fig. 4B). This might be due to the muscle gain as mice grew up. In histological evaluation, no abnormal changes in renal tubule and glomerulus were observed in the HSA dimer group (Fig. 4C). These results indicate that HSA dimer has no deleterious effect on renal function.

Plasma Clinical Chemistry and Histology for Evaluation of Muscle, Heart and Lungs CK was measured as a marker of muscle injury including skeletal muscle and myocardium. As a result, CK levels were essentially unchanged among test samples at 28 and 56 d after the commencement of administration (Fig. 5A). It is known that CK is mainly classified into three isozymes, CK-MM, CK-BB and CK-MB, which are derived from skeletal muscle, smooth muscle and myocardium, respectively. Although the present study did not evaluate the changes of each CK isozyme level, HSA dimer is unlikely to affect all kinds of muscles and cardiac function. Furthermore, there were no difference in histology in lung among saline, HSA monomer and dimer groups (Fig. 5B), suggesting that the HSA dimer does not cause lung injury.

Conclusions HSA dimer has great potentials for a variety of applications, based on its superior characteristics to HSA monomer, such as DDS carrier and plasma expander.¹⁰ Our present study is the first report regarding safety evaluation after long-term and repeated administration of HSA dim-

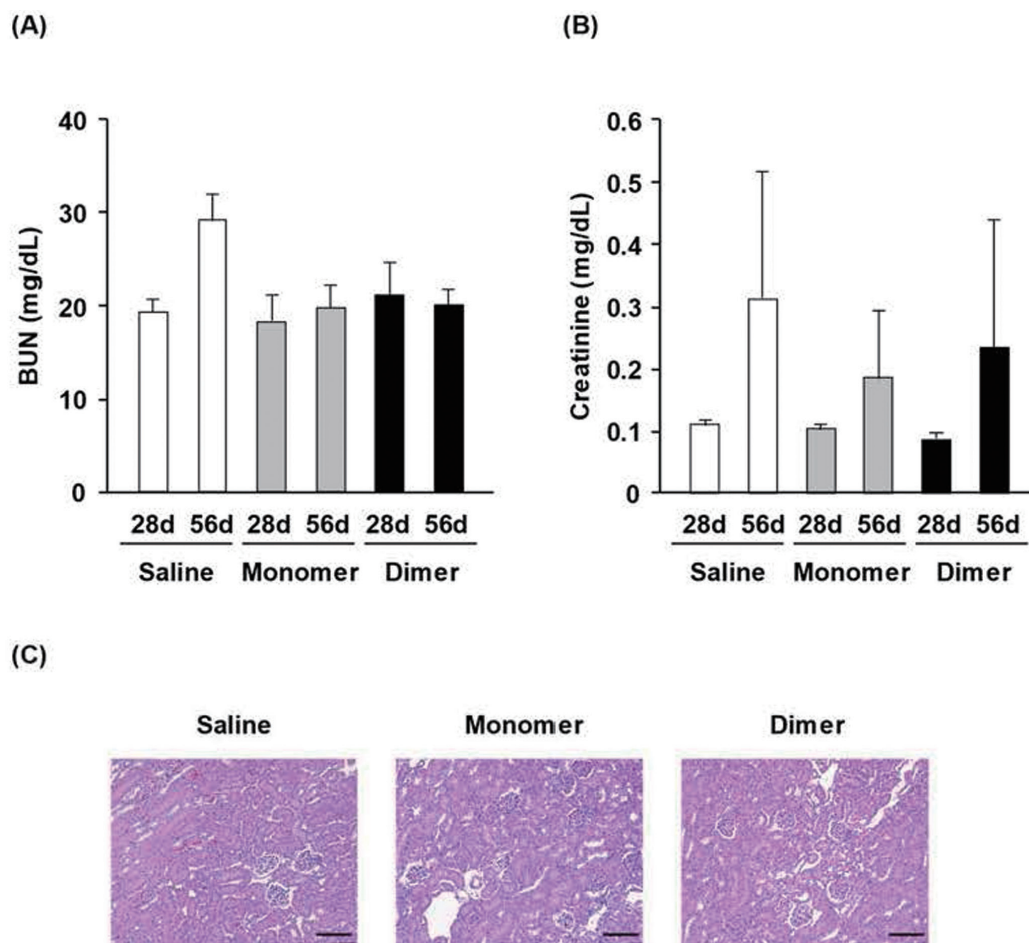


Fig. 4. Plasma Clinical Chemistries ((A) BUN and (B) Creatinine) Representing Renal Function at 28 and 56 d After the Start of Saline (Open Columns), HSA Monomer (Gray Columns) and HSA Dimer (Closed Columns) Administration to Healthy Mice. (D) Light Micrographs of Kidneys Stained with H&E

The number of mice (n) in saline (28 d), saline (56 d), HSA monomer (28 d), HSA monomer (56 d), HSA dimer (28 d) and HSA dimer (56 d) group was n=3, n=3, n=7, n=8, n=7 and n=7, respectively. Each column represents the mean \pm S.D. Scar bar represents 100 μ m.

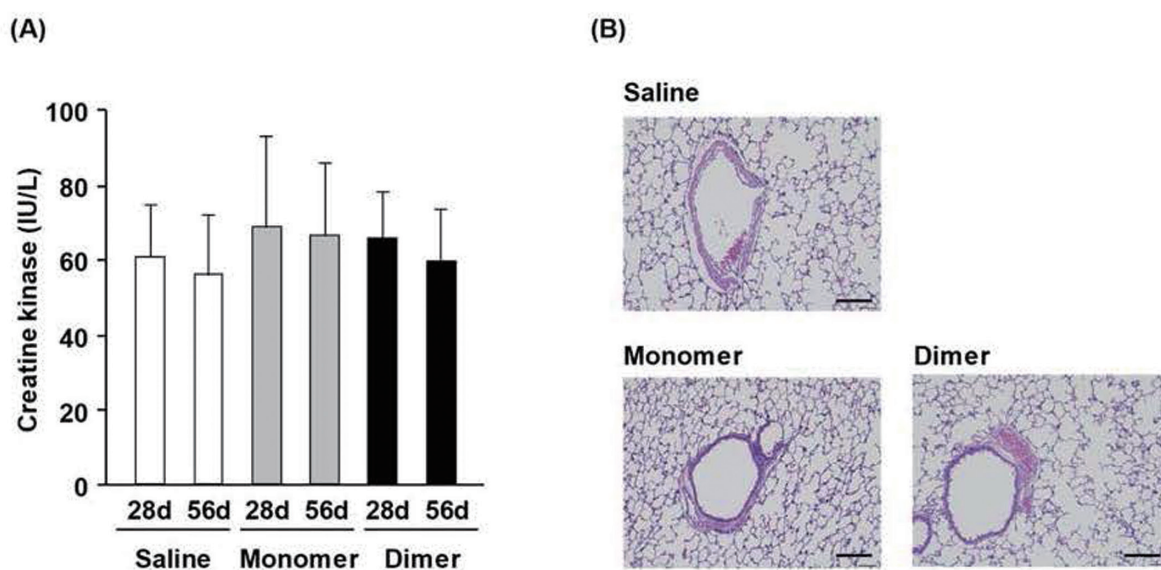


Fig. 5. (A) The Levels of CK at 28 and 56 d after the Start of Saline (Open Columns), HSA Monomer (Gray Columns) and HSA Dimer (Closed Columns) Administration to Healthy Mice. (B) Light Micrographs of Lung Stained with H&E

The number of mice (n) in saline (28 d), saline (56 d), HSA monomer (28 d), HSA monomer (56 d), HSA dimer (28 d) and HSA dimer (56 d) group was n=3, n=3, n=7, n=8, n=7 and n=7, respectively. Each column represents the mean \pm S.D. Scar bar represents 100 μ m.

er in rodent. The results of this study indicated that no adverse effects were observed in terms of physiological responses, hematology, plasma biochemical analyses and histological evaluations. The results obtained in present study provide basic information for the development of HSA dimer as clinical preparations.

Conflict of interest The authors declare no conflict of interest.

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