An NDT Study of a Boron Tracedrug UTX-51 for Glycated BSA as an AGE Model

RYU TADA[1], YOSHIHIRO UTO[1], SHIN-ICHIRO MASUNAGA[2], YUKO KINASHI[2], KOJI ONO[2] and HITOSHI HORI[1]

[1]*Department of Life System, Institute of Technology and Science, Graduate School, The University of Tokushima, Tokushima, Japan;*
[2]*Research Reactor Institute, Kyoto University, Kumatoricho, Osaka, Japan*

Abstract. *Background: Conventional therapies for diseases that are associated with protein aggregation typically prevent rather than clear protein aggregates. We have proposed neutron dynamic therapy (NDT) as a physical clearance therapy for protein aggregates. Advanced glycation end-products (AGEs), which are aggregated proteins, have been implicated in diabetes, Alzheimer's, and heart disease. Herein, we report the use of the boron tracedrug UTX-51, under thermal neutron irradiation, as an NDT for the targeted clearance of glycated bovine serum albumin (Gly-BSA), a model of AGEs. Materials and Methods: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed to detect Gly-BSA decomposition by thermal neutron irradiation treated with UTX-51. Results: The combination of UTX-51 with neutron irradiation showed a decrease in band intensity of Gly-BSA. Conclusion: We present our NDT strategy, which has been used for the targeted clearance of Gly-BSA, suggesting that NDT with boron tracedrugs can be used for the treatment of AGEs-related disease.*

Proteins are constantly 'dancing', are stabilized or modified by other proteins in their vicinity. Under conditions of stress, including oxidation, pH fluctuations, or glycation, the equilibrium is irreversibly lost and proteins become denatured and lose their functionality (1). Denatured proteins form aggregates those organize into complex assemblies, such as those observed in amyloid-β and tau proteins in Alzheimer's disease (2), α-synuclein in Parkinson's disease (3), and p53 in cancer (4). Similar to prion proteins (5), these aggregated proteins are proposed to be self-propagating (6), causing symptoms to progress until these proteins becoame detectable. Clearance therapy, which is focused on clearing, reducing, and removing aggregation-prone proteins, is an important therapeutic strategy.

Advanced glycation end-products (AGEs) are characterized as aggregated proteins (7). As shown in Figure 1, AGEs are very large molecules produced by serum proteins that have reacted with sugars non-enzymatically. AGEs are detected in numerous tissues in diabetic patients who cannot control their blood sugar levels, including the retina, kidney, and heart. Increased AGE levels are associated with the progression of Alzheimer's disease, cardiac dysfunction, and cancer symptoms (8).

Several drugs, such as aminoguanidine, pyridoxamine, and azilsartan, have been developed for the treatment for AGEs. These drugs inhibit AGE formation through their carbonyl trap activity, and blood pressure-lowering activity (Figure 2) (9, 10). These drugs have side-effects and their AGE-inhibiting effects have been proved to be limited in experimental models of diabetes (10). ALT-711, the only AGE crosslink-breaking drug tested in advanced clinical trials, reportedly has limited effects (11) and is an ineffective treatment (12). Most AGE inhibitors are thought to be ineffective because of their limited effect on existing denatured proteins.

To promote the elimination of aggregated and denatured proteins, we have developed a neutron dynamic therapy (NDT) (13, 14). NDT exploits the neutron-capture ability of a stable isotope of boron (B-10), which has been used in boron neutron capture therapy (15, 16). By introducing B-10 into a drug scaffold, we have successfully developed boron tracedrugs possessing molecular disrupting and tracing abilities. Herein, we evaluated the ability of our NDT to target glycated bovine serum albumin (Gly-BSA) to

Correspondence to: Dr. Hitoshi Hori and Dr. Yoshihiro Uto, Department of Life System, Institute of Technology and Science, Graduate School, The University of Tokushima, Minamijosanjimacho-2, Tokushima 770-8506, Japan. Tel: +81 886567514, Fax: +81 886569164, e-mail: hori@bio.tokushima-u.ac.jp, hitoshi.hori@gmail.com; uto@bio.tokushima-u.ac.jp

Key Words: Neutron dynamic therapy, Gly-BSA, AGEs, boron tracedrug, UTX-51, curcuminoid.

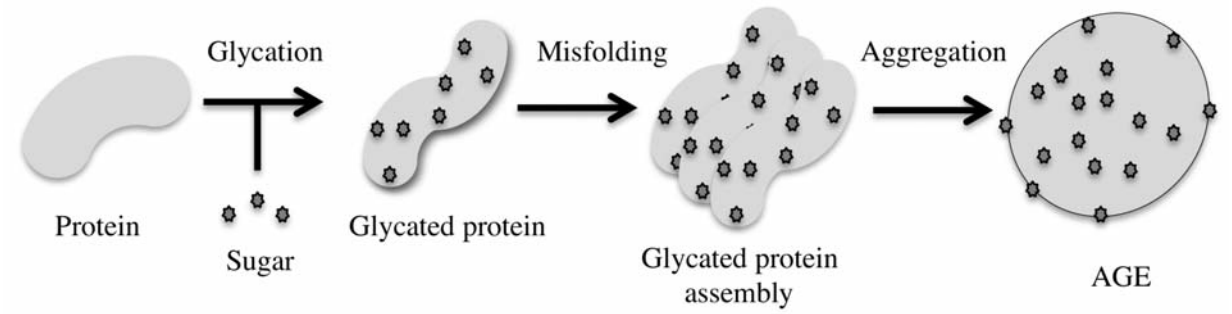

Figure 1. *Formation of glycated proteins and advanced glycation end-products (AGEs). In living tissues, the glycation of a protein with sugars induces its denaturation and the formation of aggregates (AGEs).*

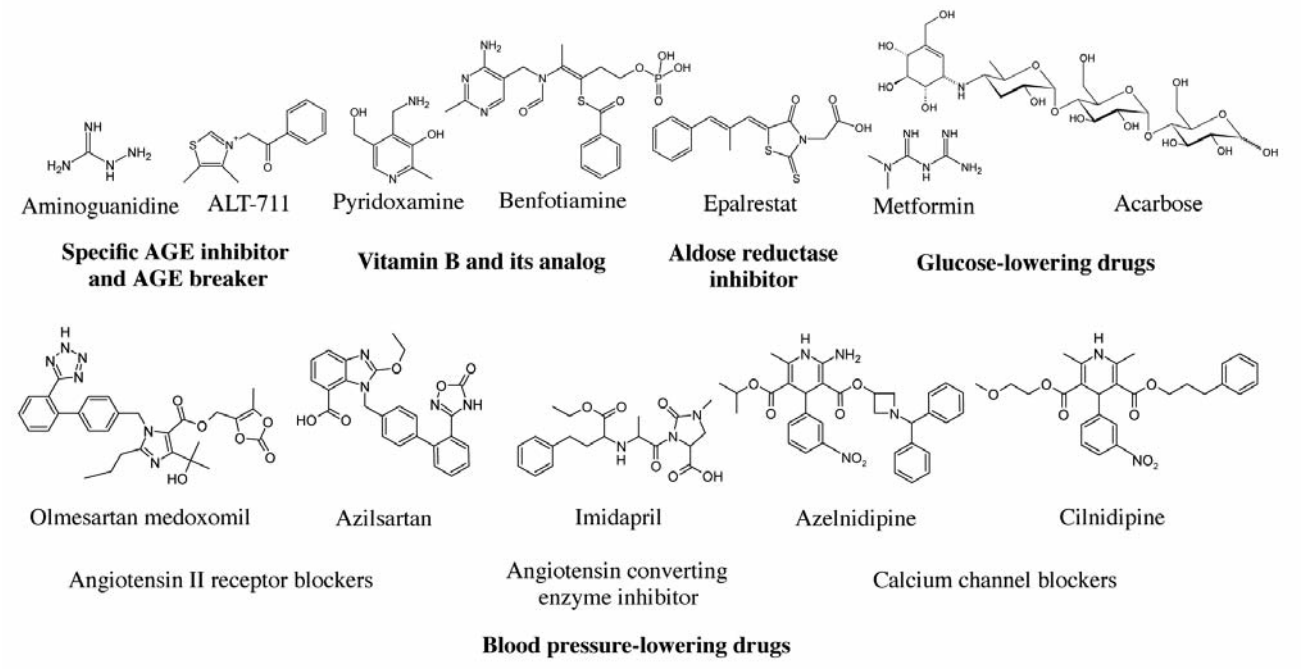

Figure 2. *The chemical structures of conventional advanced glycation end product drugs.*

determine whether the boron tracedrug UTX-51 is an effective treatment for AGEs.

Materials and Methods

Materials. All chemicals were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), Wako Pure Chemical Industries Ltd. (Osaka, Japan), and Nacalai Tesque INC. (Kyoto, Japan). Nuclear magnetic resonance (NMR) ^{1}H spectra were obtained using a JNM-EX400 spectrometer (Jeol Ltd., Tokyo, Japan) at 400 MHz. Solvents were evaporated under reduced pressure on a rotary evaporator. Thin-layer chromatography was performed on glass-backed silica gels (Merck 60 F_{254}) and components were visualized using ultraviolet (UV) light. Column chromatography was performed using KANTO Chemical silica gel 60 N (spherical neutral) 40-50 μm.

Preparation of Gly-BSA. We prepared Gly-BSA, which served as our AGE model, by using the thermal glycation method (17). BSA (2 mg; fatty acid-free; Wako Pure Chemical Industries Ltd.), 0.5 M glucose, and 0.1 M lysine, each dissolved in 0.2 M sodium phosphate buffer (SPB buffer 1.5 ml) (pH 7.5), were incubated at 50˚C for 4 days to produce Gly-BSA. The control samples of BSA and BSA with 0.1 M glucose were also incubated under similar conditions.

Analysis. We confirmed the glycation of BSA by measuring emissions at 440 nm upon excitation at 370 nm by using the Tecan Infinite M200 Microplate reader (Tecan group Ltd., Männedorf, Switzerland).

Synthesis of UTX-51. Curcumin (1,000 mg, 2.7 mmol; Tokyo Chemical Industry Co. Ltd.) was suspended in diethyl ether (6 ml) under an atmosphere of nitrogen. Boron trifluoride etherate (1 ml,

8.1 mmol) was added drop-wise with stirring at room temperature for 1 h. The solvent was evaporated. The residue was purified using silica gel column chromatography (Eluents: hexane:EtOAc=10:1) to generate UTX-51 as a red solid (1094 mg, 97% yield) (18).

NDT experiment and evaluation. Samples for NDT analysis were prepared using Gly-BSA and native BSA. The Gly-BSA solution was diluted in 0.2 M SPB (660 ng/μl) and was added 100 μl to Teflon tubes. UTX-51 was stoichiometrically diluted in microtubes to 2 μg/50 μl, 20 μg/50 μl, and 200 μg/50 μl by using dimethyl sulfoxide (DMSO). To each Teflon tube containing Gly-BSA-SPB solution, 50 μl of UTX-51-DMSO solution was added. Control samples of Gly-BSA alone were prepared. BSA was dissolved in SPB buffer (330 μg/ml) and 100 μl of this solution was transferred to each Teflon tube. To each Teflon tube containing BSA-SPB solution, 25 μl of UTX-51-DMSO solution was added. A sample containing 200 μg of UTX-51-DMSO solution was added to BSA (66 μg/100 μl SPB buffer). Control samples of BSA alone were prepared.

All samples were adjusted using SPB buffer to prepare a solution of 200 μl. A subset of the samples were irradiated. Samples were irradiated using thermal neutrons at the Kyoto University Research Reactor Institute. Irradiation conditions were as follows: time, 45 min; and absorbance dose, 0.31 Gy in the presence of B-10 (100 nmol). Absorbance dose was measured by taping the gold at the front and back sides of the Teflon tubes.

After irradiation, we evaluated the integrity of target molecules by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue (CBB). Band intensities were measured and compared using Just TLC software (Sweday., Lund, Sweden).

Results

Preparation of Gly-BSA. BSA samples incubated in the absence of glucose at 50˚C showed no change in their fluorescence intensity at 440 nm, whereas BSA incubated with glucose and lysine showed high fluorescence intensity at 440 nm. As shown in Figure 3, the fluorescence spectrum showed BSA incubated with glucose and lysine had a higher intensity than that of BSA in the presence of glucose, indicating protein glycation had occurred, producing Gly-BSA (19).

NDT experiment and analysis. We evaluated the ability of UTX-51 to disrupt Gly-BSA and native BSA by irradiating samples containing UTX-51 and the target proteins with thermal neutrons. We then compared the resultant protein levels by using SDS-PAGE (Figures 4 and 5). As a control, two samples containing Gly-BSA and native BSA were left unirradiated (lane 2). Gly-BSA samples irradiated using thermal neutrons in the presence of 500 nmol UTX-51 (100 nmol B-10) had a band intensity that was 33% lower than that of the control (lane 6, Figure 4), while native BSA had a band intensity that was 54% less than that of the control (lane 6, Figure 5). These data demonstrated the NDT effects of the boron tracedrug molecules embedded in the surface of Gly-BSA.

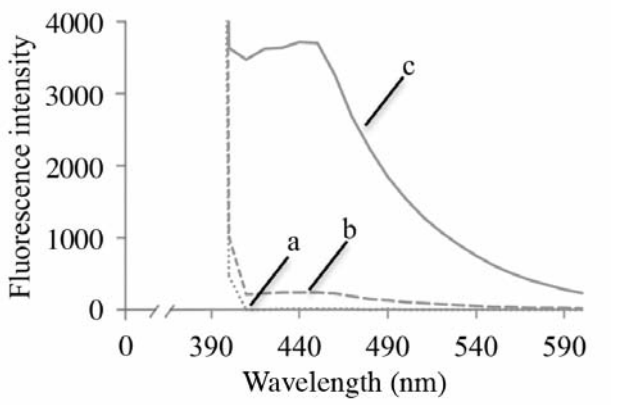

Figure 3. *Fluorescence intensity spectra for the reaction containing bovine serum albumin (BSA) and glucose. Curve a: BSA incubated at 50˚C for 4 days. Curve b: BSA and D-glucose incubated at 50˚C for 4 days. Curve c: BSA, D-glucose, and L-lysine incubated at 50˚C for 4 days.*

Discussion

Herein, we confirmed the effectiveness of the boron tracedrug UTX-51 as an NDT to decompose Gly-BSA and native BSA under thermal irradiation. These results suggest that other denatured proteins implicated in refractory diseases such as amyloid-β and tau proteins in Alzheimer's disease (2), α-synuclein in Parkinson's disease (3), and p53 in cancer (4) can be destroyed in this manner. UTX-51 (500 nmol) was less destructive towards Gly-BSA than native BSA, supporting the results of other studies that showed that aggregated proteins tend to be resistant to proteinase K (20). Thus, AGEs such as Gly-BSA assemblies might be resistant to clearance by immune cells and progressively accumulate in the body.

Our novel NDT involved the use of innovative boron tracedrugs that are markedly different from conventional therapies such as chemotherapy and immunotherapy. AGE drugs that have been tested in clinical trials can be classified into seven types: (i) specific AGE inhibitors and breakers such as aminoguanidine and ALT-711; (ii) B vitamins and analogs; (iii) the aldose reductase inhibitor epalrestat; (iv) glucose-lowering drugs such as metformin; (v) blood pressure-lowering drugs such as angiotensin II receptor blockers, angiotensin-converting enzyme inhibitor, and calcium channel blockers; (vi) lipid-lowering drugs such as atorvastatin; and (vii) anti-rheumatic agents such as methotrexate (10). In clinical trials, almost all these drugs were shown to have limited effects or to be ineffective treatments for AGEs. In particular, aminoguanidine showed an unfavorable-perceived in the risk-to-benefit ratio (21). Therefore, these drugs must be used long-term to attain results (10). With the exception of AGE breakers, these drugs are expected to have preventive effects such as anti-glycation, anti-oxidation, metal chelation,

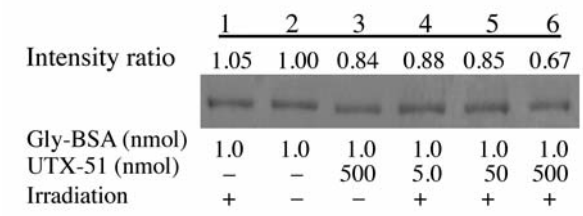

Figure 4. *Evaluation of UTX-51 as an neutron dynamic therapy for glycated bovine serum albumin (Gly-BSA) under neutron irradiation. Each lane contained 1 nmol Gly-BSA. Lane 1: Gly-BSA with irradiation; lane 2: Gly-BSA without irradiation; lane 3: UTX-51 (500 nmol) + Gly-BSA without irradiaton; lane 4: UTX-51 (5 nmol) + Gly-BSA with irradiation; lane 5: UTX-51 (50 nmol) + Gly-BSA with irradiation; lane 6: UTX-51 (500 nmol) + Gly-BSA with irradiation. Gly-BSA treated with UTX-51 on SDS-PAGE gel was stained with Coomassie Brilliant Blue. Band intensities were measured and compared using SWEDAY Just TLC software. The band intensity in lane 2 was set at 1.00 as a control. B-10:Gly-BSA=1:1 in lane 4, B-10:Gly-BSA=10:1 in lane 5, B-10:Gly-BSA=100:1 in lane 6.*

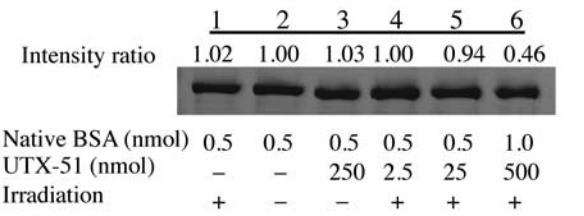

Figure 5. *Evaluation of UTX-51 as an neutron dynamic therapy for native bovine serum albumin (BSA) under neutron irradiation. Lane 1: native BSA (0.5 nmol) with irradiation; lane 2: native BSA (0.5 nmol) without irradiation; lane 3: UTX-51 (250 nmol) + native BSA (0.5 nmol) without irradiaton; lane 4: UTX-51 (2.5 nmol) + native BSA (0.5 nmol) with irradiation; lane 5: UTX-51 (25 nmol) native BSA (0.5 nmol) with irradiation; lane 6: UTX-51 (500 nmol) + native BSA (1.0 nmol) with irradiation. Native BSA treated with UTX-51 on SDS-PAGE gel was stained with Coomassie Brilliant Blue. Band intensities were measured and compared using SWEDAY Just TLC software. The band intensity in lane 2 was set at 1.00 as a control. B-10:native BSA=1:1 in lane 4, B-10:native BSA=10:1 in lane 5, B-10:native BSA=100:1 in lane 6.*

renal protection, and polyol metabolic inhibition, but they have no effect on existing denatured proteins. In preventative therapies, a regular administration of the drugs is necessary, imposing a strain on patients.

NDT, on the other hand, has the ability to completely destroy denatured proteins in tissues by using high-energy α-particles produced from B-10-captured thermal neutrons. Thermal neutron irradiation in the presence of NDT drugs requires significantly less time than the aforementioned conventional drug-based AGE therapies.

Our NDT strategy has other advantages. i) NDT of low-cost, requiring only B-10; and unlike conventional drugs, expensive production processes can be avoided. ii) No personalization is required; it is an all-purpose and patient-friendly treatment for a variety of diseases. iii) Unnecessary damage to surrounding normal tissues is minimized because NDT uses thermal neutrons with low energy to irradiate B-10 atoms, forming a functional drug. iv) NDT requires few to no pharmacokinetic and pharmacodynamic studies, which are typically necessary to develop new drugs.

To the best of our knowledge, our present study is the first to report the destruction of a denatured protein, Gly-BSA, by using energy generated from B-10 captured neutrons. We believe that our physically-powered NDT using boron tracedrugs is a next-generation standard therapy that can replace conventional chemistry-based therapies. However, for NDT to become an established therapy, a compact neutron generator for NDT must be developed.

In conclusion, we present our NDT strategy, which has been used for the targeted clearance of Gly-BSA in a model of AGE. Our results suggest that NDT with boron tracedrugs can be used for the treatment of diabetes, Alzheimer's disease, and heart disease.

References

1 Hartl FU, Bracher A and Hayer-Hartl M: Molecular chaperones in protein folding and proteostasis. Nature *475*: 324-332, 2011.
2 Ittner LM and Götz J: Amyloid-β and tau–a toxic pas de deux in Alzheimer's disease. Nature *12*: 67-72, 2011.
3 Uversky VN: Neuropathology, biochemistry, and biophysics of α-synuclein aggregation. J Neurochem *103*: 17-37, 2007.
4 Rangel LP, Costa DC, Vieira TC and Silva JL: The aggregation of mutant p53 produces prion-like properties in cancer. Prion *8*: 75-84, 2014.
5 Prusiner SB: Biology and genetics of prions causing neurodegeneration. Annu Rev Genet *47*: 601-623, 2013.
6 Jucker M and Walker LC: Self-propagation of pathogenic protein aggregates in neurodegenerative diseases. Nature *501*: 45-51, 2013.
7 Pietropoli D, Monaco A, Del Pinto R, Cifone MG, Marzo G and Giannoni M: Advanced glycation end-products: possible link between metabolic syndrome and periodontal diseases. Int J Immunopathol Pharmacol *25*: 9-17, 2012.
8 Willis MS, and Patterson C: Proteotoxicity and cardiac dysfunction–Alzheimer's disease of the heart? N Engl J Med *368*: 455-464, 2013.
9 Schalkwijk CG and Miyata T: Early- and advanced non-enzymatic glycation in diabetic vascular complications: the search for therapeutics. Amino acids *42*: 1193-1204, 2012.
10 Engelen L, Stehouwer CD and Schalkwijk CG: Current therapeutic interventions in the glycation pathway: evidence from clinical studies. Diabetes Obes Metab *15*: 677-689, 2013.
11 Sell DR and Monnier VM: Molecular basis of arterial stiffening: role of glycation – A mini review. Gerontology *58*: 227-237, 2012.
12 Hartog JW, Willemsen S, van Veldhuisen DJ, Posma JL, van Wijk LM, Hummel YM, Hillege HL and Voors AA; Effects of alagebrium, an advanced glycation endproduct breaker, on exercise tolerance and cardiac function in patients with chronic heart failure. Eur J Heart Fail *13*: 899-908, 2011.

13 Hori H, Uto Y and Nakata E: Medicinal electronomics bricolage design of hypoxia-targeting antineoplastic drugs and invention of boron tracedrugs as innovative future-architectural drugs. Anticancer Res *30*: 3233-3242, 2010.
14 Hori H, Uto Y and Nakata E: Boron tracedrugs challenge for neutron dynamic therapy. Anticancer Res *32*: 2235-2239, 2012.
15 Barth RF, Vicente MG, Harling OK, Kiger WS 3rd, Riley KJ, Binns PJ, Wagner FM, Suzuki M, Aihara T, Kato I and Kawabata S: Current status of boron neutron capture therapy of high-grade gliomas and recurrent head and neck cancer. Radiat Oncol *7*: 146, 2012.
16 Moss RL: Critical review, with an optimistic outlook, on boron neutron capture therapy (BNCT). Appl Radiat Isot *88*: 2-11, 2014.
17 Bhatwadekar AD and Ghole VS: Rapid method for preparation of an AGE-BSA standard calibrator using thermal glycation. J Clin Lab Anal 19: 11-15, 2005.
18 Nakata E, Koizumi M, Yamashita Y, Onaka K, Sakurai Y, Kondo N, Ono K, Uto Y and Hori H: Design, synthesis and destructive dynamic effects of BODIPY-containing and curcuminoid boron tracedrugs for neutron dynamic therapy. Anticancer Res *31*: 2477-2481, 2011.
19 Monnier VM, Kohn RR and Cerami A: Accelerated age-related browning of human collagen in diabetes mellitus. Proc Natl Acad Sci USA *81*: 583-587, 1984.
20 Yuan J, Xiao X, McGeehan J, Dong Z, Cali I, Fujioka H, Kong Q, Kneale G, Gambetti P and Zou WQ: Insoluble aggregates and protease-resistant conformers of prion protein in uninfected human brains. J Biol Chem *281*: 34848-34858, 2006.
21 Suji Gand Sivakami S: DNA damage by free radical production by aminoguanidine. Ann NY Acad Sci *1067*: 191-199, 2006.

Received April 4, 2014
Revised June 10, 2014
Accepted June 11, 2014