

Enhancement of HIV-1 Tat fusion protein transduction efficiency by bog blueberry anthocyanins

Sun Hwa Lee^{1,#}, Hoon Jae Jeong^{1,#}, Dae Won Kim^{1,#}, Eun Jeong Sohn¹, Mi Jin Kim¹, Duk Soo Kim², Tae Cheon Kang³, Soon Sung Lim⁴, Il Jun Kang⁴, Sung-Woo Cho⁵, Kil Soo Lee¹, Jinseu Park¹, Won Sik Eum^{1,*} & Soo Young Choi^{1,*}

¹Department of Biomedical Science & Research Institute of Bioscience and Biotechnology, Hallym University, Chunchon 200-702,

²Department of Anatomy, College of Medicine, Soonchunhyang University, Cheonan 330-090, ³Department of Anatomy & Neurobiology, College of Medicine, Hallym University, Chunchon 200-702, ⁴Department of Food Science and Nutrition & RIC Center, Hallym University, Chunchon 200-702, ⁵Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, Seoul 138-736, Korea

Though protein transduction domains (PTDs) are well known for the delivery of exogenous therapeutic proteins into living cells, the overall low efficiency of transduction is a serious obstacle. We investigated the effect of bog blueberry anthocyanins (BBA) on protein transduction efficiency and found that BBA enhanced the transduction efficiencies of Tat-SOD fusion protein into HeLa cells and mice skin. The enzymatic activities in the cells and skin tissue in the presence of BBA were markedly increased compared to controls. Further, BBA did not demonstrate any cell toxicity at various concentrations. Although the mechanism is not fully understood, we suggest that BBA might alter the conformation of the membrane, which would indicate that BBA can be used as a protein transduction enhancer for the efficient delivery of therapeutic proteins for a variety of disorders. [BMB reports 2010; 43(8): 561-566]

INTRODUCTION

The size and biochemical properties of therapeutic proteins limit their ability to enter cells (1). Protein transduction technology based on small domains called protein transduction domains (PTDs) and cell penetrating peptides (CPPs) has been used to deliver exogenous protein into cultured cells and animal models (2-7). Many research groups have demonstrated the efficacy of protein transduction technology for protein therapy strategies as well as drug delivery, although the exact mechanism of transduction remains unclear (8-19). Although various proteins have been developed for protein therapy, protein transduction technology has several limitations such as transduction efficiency. Several studies have demonstrated how to

overcome those limitations (20-22). In preliminary studies, we screened a number of natural products that have the ability to enhance the transduction efficiency of therapeutic fusion proteins. Among the natural products tested, we found that bog blueberry anthocyanins (BBA) is an efficient protein transduction enhancer.

Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion and hydroxyl radicals are inevitably produced as the side effects of normal cellular processes and macromolecular damage in cells. Cu,Zn-superoxide dismutase (SOD) is an antioxidant enzyme that catalyzes the dismutation of two superoxide anions into oxygen and hydrogen peroxide, and it is thought to protect cells against oxidative damage (23). For these reasons it has been utilized as a therapeutic protein for protection against ROS-induced damage (24, 25).

Anthocyanins are important plant pigments with several biological functions, including antioxidant and anti-inflammatory effects as well as prevention of chronic disease (26-28). Anthocyanins are composed of two groups derived from the flavylum. The major anthocyanidins such as glucose, galactose, arabinose, xylose and rhamnose are common sugars (29) while common anthocyanidins such as delphinidin, cyaniding, petunidin, peonidin, pelargonidin and malvidin are found in fruits, vegetables and berries. Generally, berries are harvested for household consumption and commercial sale as well as for use in folk medicine throughout Asian and Europe (30).

Although a number of anthocyanins have been studied, the precise manner in which they change the conformation of the cell membrane and skin tissue is unknown. Therefore, we investigated whether anthocyanins could enhance the transduction of the cell-permeable antioxidant enzyme Tat-SOD into cells and skin tissue.

RESULTS AND DISCUSSION

Identification and cell toxicity of BBA

A number of anthocyanins have been identified from edible blueberry, including bog blueberry (*Vaccinium uliginosum* L) (Korean name: Deol-Juguk) (30-34). In this study, we examined

*Corresponding author. Tel: 82-33-248-2112; Fax: 82-33-248-3201; E-mail: sychoi@hallym.ac.kr and wseum@hallym.ac.kr

#These authors equally contributed to this work.

Received 9 June 2010, Accepted 22 July 2010

Keywords: Bog blueberry anthocyanins, Protein therapy, Protein transduction efficiency, Tat fusion protein

the effects of the anthocyanins of bog blueberry (BBA) on the transduction of Tat-SOD into HeLa cells and animal skin. As shown in Fig. 1, BBA was identified as cyanidine-3-glucoside, petunidin-3-glucoside, malvidine-3-glucoside, delphinidin-3-glucoside and delphinidine-3-arabinoside.

To determine the cytotoxic effects of BBA on HeLa cells, the cells were treated with various (100-1000 ng/ml) concentrations of BBA and incubated for 12 h. As shown in Fig. 2, BBA did not affect cell viability at various concentrations. These results indicate that BBA was not cytotoxic. Others studies have shown that cyanidine-3-glucoside, petunidin-3-glucoside, malvidine-3-glucoside and delphinidin-3-glucoside are present in red wine where they act as natural colorants. In addition, human consumption of blueberry juice had no negative effects (35-37).

Endogenous SOD activity was not significantly changed when cells were treated with BBA for 12 h (data not shown).

Effects of BBA on Tat-SOD transduction into cells and skin

We reported that Tat-SOD fusion proteins are transduced into HeLa cells, where they have a protective effect against oxidative stress (38). In recent decades, protein transduction technologies have been developed for therapeutic purposes (2). In addition, these technologies have been used to successfully transduce a number of different therapeutic proteins both *in vitro* and *in vivo* (4). However, protein transduction technology has problems related to transduction efficiency, which make it inadequate for therapeutic applications. Therefore, increasing the transduction efficiency is a very important obstacle that must be overcome for the development of protein

therapy technology.

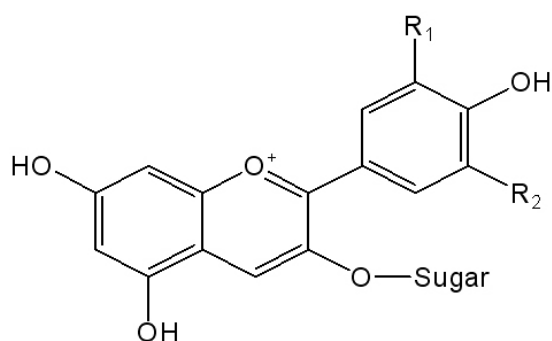
In the present study, we investigated the effects of BBA on the transduction of Tat-SOD into mammalian cells via Western blotting using anti-histidine antibody and enzyme assays. Various concentrations of Tat-SOD (0.5-3 μ M) fusion proteins were added to a culture media of HeLa cells for 1 h. Further, Tat-SOD fusion proteins were added to the culture media of HeLa cells at a concentration of 3 μ M for various times (10-60 min). As shown in Fig. 3A, the levels of transduced Tat-SOD fusion proteins in cultured HeLa cells significantly increased in a dose- and time-dependent manner when the cells were pre-incubated with BBA for 12 h.

The enzymatic activities of the transduced Tat-SOD fusion proteins must be maintained if any therapeutic application is desired. Therefore, we determined the dismutase activities of SOD in HeLa cells treated with Tat-SOD and BBA. As shown in Fig. 3B, SOD activity markedly increased in a dose- and time-dependent manner in cells treated with BBA. These results indicate that BBA did not impact cell growth and increased Tat-SOD fusion protein transduction efficiency.

As positive control experiments, we examined whether or not BBA increased the transduction efficiency of a control protein such as Tat-GFP. As shown in Fig. 3C, BBA also enhanced the transduction efficiency of Tat-GFP.

Next, we examined the effect of BBA on the transduction of Tat-SOD fusion protein into mice skin by immunohistochemistry and SOD activity. As shown in Fig. 4, the transduction efficiency and SOD enzyme activity were markedly increased by the presence of BBA. In addition, the levels of enzyme activities in skin increased approximately 3-4 fold compared to that treated solely with Tat-SOD. These results demonstrate that BBA enhanced the transduction efficiency of Tat-SOD fusion protein into cells and skin tissue.

Recent studies have demonstrated various methods to enhance transduction efficiency (20, 21). Wang et al. (2010) de-



	R1	R2	Sugar
Cyanidin-3-glucoside	OH	H	Glucose
Petunidin-3-glucoside	OCH3	OH	Glucose
Malvidin-3-glucoside	OCH3	OCH3	Glucose
Delphinidin-3-glucoside	OH	OH	Glucose
Delphinidin-3-arabinoside	OH	OH	Arabinose

Fig. 1. Structure of bog blueberry anthocyaninins (BBA).

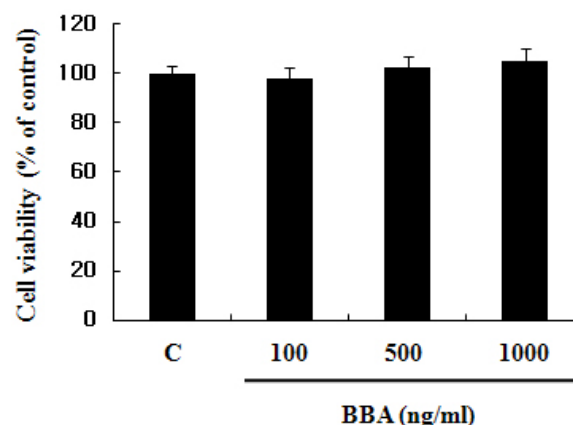


Fig. 2. Effect of BBA on cell viability. Cells were incubated with BBA (100-1,000 ng/ml) for 12 h. Cell viabilities were estimated by colorimetric assay using MTT.

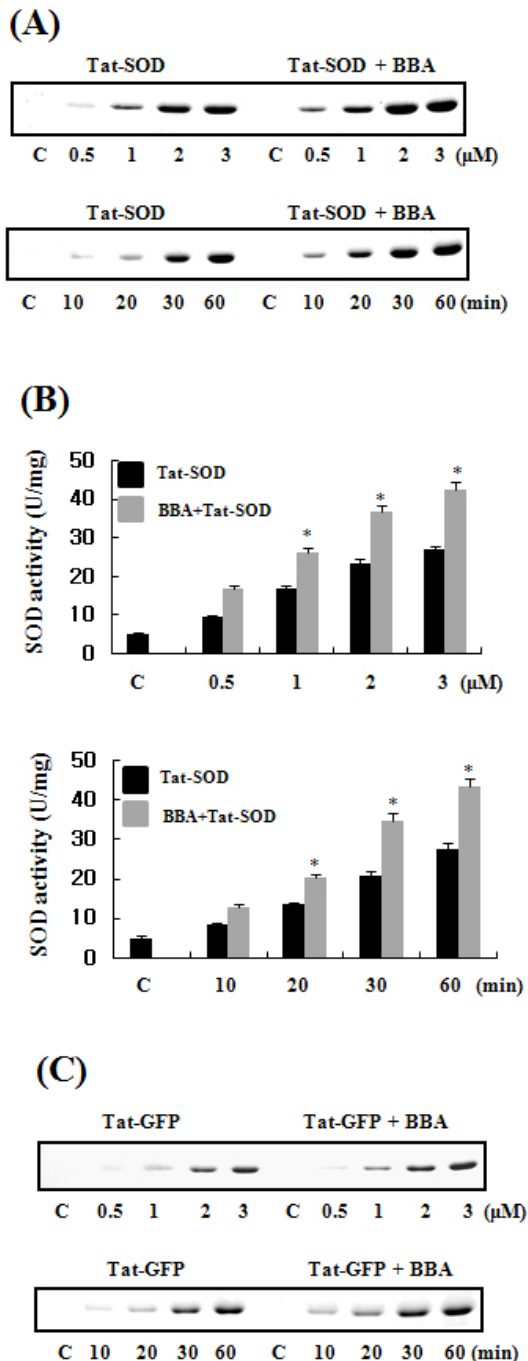


Fig. 3. Effect of Tat-SOD transduction (A), enzyme activity (B) and Tat-GFP (C) in HeLa cells. Dose (0.5-3 μM) and time (10-60 min)-dependent transduction of Tat-fusion protein into cultured HeLa cells. Cells were pretreated with BBA (1,000 ng/ml) for 12 h. Transduced Tat fusion proteins and enzyme activity were analyzed by Western blotting and by measuring specific enzyme activities. *P < 0.01 compared with treated with Tat-SOD.

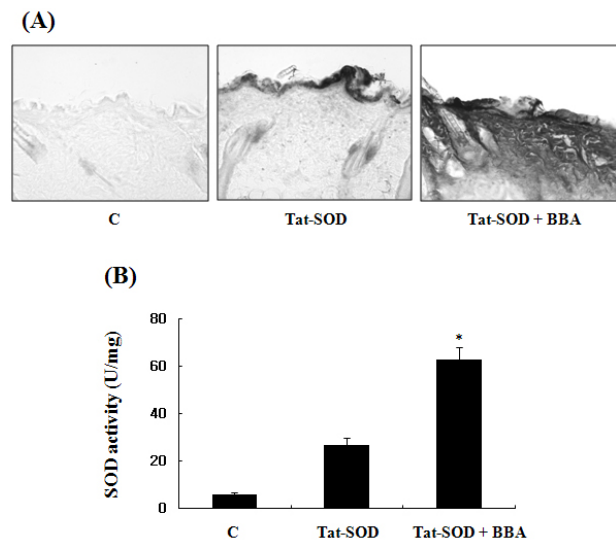


Fig. 4. Immunohistochemical analysis of animal skin transduced with Tat-SOD proteins. Tat-SOD (50 μg) was applied topically onto a shaved area of mouse dorsal skin for 1 h. Frozen sections of the skin tissues were immunostained with rabbit anti-histidine IgG, as described in Materials and Methods. The sections were visualized with 3,3'-diaminobenzidine and observed using an Axioscope microscope (A). Transduction efficiencies were analyzed by measuring the specific enzyme activities of the skin tissue (B). *P < 0.01 compared with treated with Tat-SOD.

monstrated that lower concentrations of DMSO markedly improve Tat fusion protein transduction into cells without cytotoxic effects or perforation of the membrane. In addition, they suggested that application of DMSO as a transduction enhancer is a viable strategy for increasing transduction efficiency. However, high concentrations of DMSO have a repressive effect on cell growth and cytotoxicity, depending on the cell lines in question. Lim et al. (2010) demonstrated that therapeutic protein genes fused with different protein transduction domains (PTDs) such as HIV-1 Tat and 11-arginin tend to overcome low transduction efficiency. Although transduction proteins show protective effects in cells, the protective effects afforded by PTDs are different. In this study, we used a natural product as a transduction efficiency enhancer. Anthocyanins from edible bog blueberries (BBA) have various biological effects on human and animal diseases. We have shown that BBA is non-toxic to cell's suggesting it may be used as a protein transduction enhancer without any side effects related to toxicity.

In summary, we demonstrated that BBA enhanced the transduction of Tat-SOD into cell and skin tissue, which supports BBA as an efficient strategy for the delivery of therapeutic proteins. However, the detailed mechanism by which BBA affects transduction requires further study.

MATERIALS AND METHODS

Materials

ICR mice (6-8 weeks) were purchased from the Experimental Animal Center, Hallym University, Chunchon, Korea. The animals used in this experiment were treated according to the "Principles of Laboratory Animal Care" (NIH Publication No. 86-23) approved by the Hallym Medical Center Institutional Animal Care and Use Committee. Cell permeable Tat-SOD was expressed and purified as described previously (38). Rabbit anti-histidine polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and all other chemicals and reagents were of the highest analytical grade available. Edible bog blueberry (*Vaccinium uliginosum* L., Korean name "Deol-Jugk") was collected from BeagDu Mountain, North Korea, in August 2005. A voucher specimen was deposited in the Herbarium of the Regional Innovation Center at Hallym University, Chunchon, Korea.

Extraction and identification of bog blueberry anthocyanins

Bog blueberry anthocyanins (BBA) were extracted and identified as previously described (39). Fresh bog blueberries (100 g) were juiced in 100 ml on water, followed by filtration using Whatman No.2 filter paper. After the filtrate was adsorbed on a Dianion HP-20 resin column and washed with water and ethanol, the anthocyanin-rich fractions were obtained. Anthocyanin was flowed through a RP YMC ODS H-80 column using a Finnigan Surveyor HPLC system (ThermoQuest, San Jose, CA) and a Finnigan LCQ Advantage IT mass spectrometer (ThermoQuest, San Jose, CA). The final compounds were confirmed using an *m/z* value following MS/MS of the *m/z* value. Five grams of anthocyanins was obtained from 100 g of fresh bog blueberry.

Effects of BBA on Tat-SOD activity and transduction

To measure Tat-SOD transduction and SOD activity, the BBA samples were dissolved in DMSO and then added to HeLa cells. HeLa cells were maintained as previously described (38). Samples were pretreated with BBA (1,000 ng/ml) for 12 h and then exposed to various concentrations of Tat-SOD for 1 h. Cells were then harvested, and the cell extracts were used for enzyme assays and Western blot analyses. SOD activity was measured by monitoring the inhibition of ferricytochrome c reduction by xanthine/xanthine oxidase reaction (40). The protein concentration was determined by the Bradford method using bovine serum albumin as a standard (41).

Western blot analysis

Sample proteins were electrophoretically transferred to a nitrocellulose membrane, after which the membrane was blocked in 5% nonfat milk in TBST buffer (TBS; 20 mM Tris, 0.2 M NaCl, pH 7.5 containing 0.05% Tween-20) for 2 h. The membrane was incubated for 1 h at room temperature with anti-histidine antibody (Santa Cruz Biotechnology, Santa Cruz, CA,

USA; dilution 1 : 400) in TBST. After washing, the membrane was incubated for 1 h with a proper secondary antibody conjugated to horseradish peroxidase diluted 1 : 10,000 in TBST. The bands were visualized by enhanced chemiluminescence according to the manufacturer's instructions (ECL; Amersham).

Measurement of BBA cytotoxicity

The cytotoxicity of BBA against HeLa cells was measured by MTT assay. The cells were treated with BBA (100-1,000 ng/ml) for 12 h, after which the culture medium and MTT solution was added and the cells incubated for 4 h. Absorbance was measured at 570 nm using an ELISA microplate reader (Labsystems Multiskan MCC/340, Labsystems, Finland).

Immunohistochemistry

Immunohistochemistry was performed as previously described (42). Animals were anesthetized with 3% isoflurane in nitrogen and oxygen. Then, control, Tat-SOD (50 µg) and BBA (100 µg/ml) combined with Tat-SOD were topically applied onto shaved areas of mouse dorsal skin for 1 h. Frozen sections of skin tissues were prepared and fixed with 4% paraformaldehyde for 10 min, followed by incubation with rabbit anti-histidine IgG (1 : 500) for 24 h at room temperature and for 1 h with biotinylated goat anti-rabbit IgG (Vector Laboratories, USA; dilution 1 : 200). The sections were visualized with 3,3'-diaminobenzidine (40 mg DAB/0.045% H₂O₂ in 100 ml PBS) and mounted on gelatin-coated slides. Immunoreactions were observed using an Axioscope microscope (Carl Zeiss, Germany).

Acknowledgements

This work was supported by a Priority Research Centers Program grant (2009-0093812) and by a Regional Research Universities Program/Medical & Bio-material Research Center grant through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology.

REFERENCES

1. Eggleton, R. D. and Davis, T. P. (1997) Bioavailability and transport of peptides and peptide drugs into the brain. *Peptides* **18**, 1431-1439.
2. Schwarze, S. R., Ho, A., Vocero-Akbani, A. and Dowdy, S. F. (1999) *In vivo* protein transduction: delivery of a biologically active protein into the mouse. *Science* **285**, 1569-1572.
3. Schwarze, S. R., Hruska, K. A. and Dowdy, S. F. (2000) Protein transduction: unrestricted delivery into all cells? *Trends Cell Biol.* **10**, 290-295.
4. Wadia, J. S. and Dowdy, S. F. (2002) Protein transduction technology. *Curr. Opin. Biotechnol.* **13**, 52-56.
5. Wadia, J. S. and Dowdy, S. F. (2003) Modulation of cellular function by TAT mediated transduction of full length proteins. *Curr. Protein Pept. Sci.* **4**, 97-104.
6. Prochiantz, J. (2000) Messenger proteins: homeoproteins, TAT and others. *Curr. Opin. Cell Biol.* **12**, 400-406.
7. Dietz, G. P. (2010) Cell-penetrating peptide technology to

- delivery chaperones and associated factors in diseases and basic research. *Curr. Pharm. Biotechnol.* **11**, 167-174.
8. Matsushita, M., Tomizawa, K., Moriawaki, A., Li, S. T., Terada, H. and Matsui, H. (2001) A high-efficiency protein transduction system demonstrating the role of PKA in long-lasting long-term potentiation. *J. Neurosci.* **21**, 6000-6007.
9. Rothbard, J. B., Garlington, S., Lin, Q., Kirschberg, T., Kreider, E., McGrane, P. L., Wender, P. A. and Khavari, P. A. (2000) Conjugation of arginine oligomers to cyclosporine A facilitates topical delivery and inhibition of inflammation. *Nat. Med.* **6**, 1253-1257.
10. Mai, J. C., Shen, H., Watkins, S. C., Cheng, T. and Robbins, P. D. (2002) Efficiency of protein transduction is cell type-dependent and is enhanced by dextran sulfate. *J. Biol. Chem.* **277**, 30208-30218.
11. Matsui, H., Tomizawa, K., Lu, Y. F. and Matsushita, M. (2003) Protein therapy: *in vivo* protein transduction by polyarginine (11R) PTD and subcellular targeting delivery. *Curr. Protein Pept. Sci.* **4**, 151-157.
12. Eum, W. S., Kim, D. W., Hwang, I. K., Yoo, K. Y., Kang, T. C., Jang, S. H., Choi, H. S., Choi, S. H., Kim, Y. H., Kim, S. Y., Kwon, H. Y., Kang, J. H., Kwon, O. S., Cho, S. W., Lee, K. S., Park, J., Won, M. H. and Choi, S. Y. (2004) *In vivo* protein transduction: biologically active intact PEP-1-superoxide dismutase fusion protein efficiently protects against ischemic insult. *Free Radic. Biol. Med.* **37**, 1656-1669.
13. Choi, S. H., Kim, S. Y., An, J. J., Lee, S. H., Kim, D. W., Ryu, H. J., Lee, N. I., Yeo, S. I., Jang, S. H., Won, M. H., Kang, T. C., Kwon, H. J., Cho, S. W., Kim, J., Lee, K. S., Park, J., Eum, W. S. and Choi, S. Y. (2006) Human PEP-1-ribosomal protein S3 protects against UV-induced skin cell death. *FEBS Lett.* **580**, 6755-6762.
14. An, J. J., Lee, Y. P., Kim, S. Y., Lee, S. H., Lee, M. J., Jeong, M. S., Kim, D. W., Jang, S. H., Yoo, K. Y., Won, M. H., Kang, T. C., Kwon, O. S., Cho, S. W., Lee, K. S., Park, J., Eum, W. S. and Choi, S. Y. (2008) Transduced human PEP-1-heat shock protein 27 efficiently protects against brain ischemic insult. *FEBS J.* **275**, 1296-1308.
15. An, J. J., Lee, Y. P., Kim, D. W., Sohn, E. J., Jeong, H. J., Kang, H. W., Shin, M. J., Kim, M. J., Ahn, E. H., Jang, S. H., Kang, J. H., Kang, T. C., Won, M. H., Kwon, O. S., Cho, S. W., Lee, K. S., Park, J., Eum, W. S. and Choi, S. Y. (2009) Transduced HSP27 protein protects neuronal cell death by enhancing FALS-associated SOD1 mutant activity. *BMB Reports* **42**, 136-141.
16. Lee, M. J., Kim, D. W., Lee, Y. P., Jeong, H. J., Kang, H. W., Shin, M. J., Sohn, E. J., Kim, M. J., Jang, S. H., Kang, T. C., Won, M. H., Min, B. H., Cho, S. W., Lee, K. S., Park, J., Eum, W. S. and Choi, S. Y. (2009) Inhibition of LPS-induced nitric oxide production by transduced Tat-arginine deiminase fusion protein in Raw 264.7 cells. *BMB Reports* **42**, 286-292.
17. Song, H. Y., Lee, J. A., Ju, S. M., Yoo, K. Y., Won, M. H., Kwon, H. J., Eum, W. S., Jang, S. H., Choi, S. Y. and Park, J. (2008) Topical transduction of superoxide dismutase mediated by HIV-1 Tat protein transduction domain ameliorates 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice. *Biochem. Pharmacol.* **75**, 1348-1357.
18. Kim, D. W., Jeong, H. J., Kang, H. W., Shin, M. J., Sohn, E. J., Kim, M. J., Ahn, E. H., An, J. J., Jang, S. H., Yoo, K. Y., Won, M. H., Kang, T. C., Hwang, I. K., Kwon, O. S., Cho, S. W., Park, J., Eum, W. S. and Choi, S. Y. (2009) Transduced human PEP-1-catalase fusion protein attenuates ischemic neuronal damage. *Free Radic. Biol. Med.* **47**, 941-952.
19. Kim, D. W., Lee, S. H., Jeong, M. S., Sohn, E. J., Kim, M. J., Jeong, H. J., An, J. J., Jang, S. H., Won, M. H., Hwang, I. K., Cho, S. W., Kang, T. C., Lee, K. S., Park, J., Yoo, K. Y., Eum, W. S. and Choi, S. Y. (2010) Transduced Tat-SAG fusion protein protects against oxidative stress and brain ischemic insult. *Free Radic. Biol. Med.* **48**, 969-977.
20. Lim, K. S., Won, Y. W., Park, Y. S. and Kim, Y. H. (2010) Preparation and functional analysis of recombinant protein transduction domain-metallothionein fusion proteins. *Biochimie* **92**, 964-970.
21. Wang, H., Zhong, C. Y., Wu, J. F., Huang, Y. B. and Liu, C. B. (2010) Enhancement of TAT cell membrane penetration efficiency by dimethyl sulphoxide. *J. Con. Rel.* **143**, 64-70.
22. Park, J., Ryu, J., Kim, K. A., Lee, H. J., Bahn, J. H., Han, K. H., Choi, E. Y., Lee, K. S., Kwon, H. Y. and Choi, S. Y. (2002) Mutational analysis of a human immunodeficiency virus type 1 Tat protein transduction domain which is required for delivery of an exogenous protein into mammalian cells. *J. Gen. Virol.* **83**, 1173-1181.
23. Bannister, J. V. and Calabrese, L. (1987) Assays for superoxide dismutase. *Methods Biochem. Anal.* **32**, 279-312.
24. Del Zoppo, G. J., Wagner, S. and Tagaya, M. (1997) Trends and future developments in the pharmacological treatment of acute ischemic stroke. *Drugs* **54**, 9-38.
25. Muzykantov, V. R., Atochina, E. N., Ischirapoulou, H., Danilov, S. M. and Fisher, A. B. (1993) Immunotargeting of antioxidant enzyme to the pulmonary endothelium. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 5213-5218.
26. Heber, D. (2004) Vegetables, fruits and phytoestrogens in the prevention of diseases. *J. Postgrad. Med.* **50**, 145-149.
27. Juranic, Z. and Zizak, Z. (2005) Biological activities of berries: from antioxidant capacity to anti-cancer effects. *Biofactor* **23**, 207-211.
28. Ghosh, D. and Konishi, T. (2007) Anthocyanins and anthocyanin-rich extracts: role in diabetes and eye function. *Asia Pac. J. Clin. Nutr.* **16**, 200-208.
29. Prior, R. L. and Wu, X. (2006) Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. *Free Radical Res.* **40**, 1014-1028.
30. Vander Kloet, S. P. and Dickinson, T. A. (1992) The taxonomy of *Vaccinium* section *Hemimyrtillus*. *Bot. Mag. Tokyo* **105**, 601-614.
31. Maatta-Riihinen, K. R., Kamal-Eldin, A., Mattila, P. H., Gonzalez-Paramas, A. M. and Torronen, A. R. (2004) Distribution and contents of phenolic compounds in eighteen Scandinavian berry species. *J. Agric. Food Chem.* **52**, 4477-4486.
32. Latti, A. K., Riihinen, K. R. and Kainulainen, P. S. (2008) Analysis of anthocyanin variation in wild populations of bilberry (*Vaccinium myrtillus* L.) in Finland. *J. Agric. Food Chem.* **56**, 190-196.

33. Lee, J., Finn, C. E. and Wrolstad, R. E. (2004) Comparison of anthocyanin pigment and other phenolic compounds of *Vaccinium membranaceum* and *Vaccinium ovatum* native to the Pacific Northwest of North America. *J. Agric. Food Chem.* **52**, 7039-7044.
34. Latti, A. K., Kainulainen, P. S., Hayirlioglu-Ayaz, S., Ayaz, F. A. and Riihinen K. R. (2009) Characterization of anthocyanins in Caucasian blueberries (*Vaccinium arctostaphylos* L.) native to Turkey. *J. Agric. Food Chem.* **57**, 5244-5249.
35. Burns, J., Gardner, P. T., Matthews, D., Duthie, G. G., Lean, M. E. and Crozier, A. (2001) Extraction of phenolics and changes in antioxidant activity of red wines during vinification. *J. Agric. Food Chem.* **49**, 5797-5808.
36. Zafra-stone, S., Yasmin, T., Bagchi, M., Chatterjee, A., Vinson, J. A. and Bagchi, D. (2007) Berry anthocyanins as novel antioxidants in human health and disease prevention. *Mol. Nutr. Food Res.* **51**, 675-683.
37. Pedersen, C. B., Kyle, J., Jenkinson, A. M., Gardner, P. T., McPhail, D. B. and Duthie, G. G. (2000) Effects of blueberry and cranberry juice consumption on the plasma antioxidant capacity of healthy female volunteers. *Eur. J. Clin. Nutr.* **54**, 405-408.
38. Kwon, H. Y., Eum, W. S., Jang, H. W., Kang, J. H., Rye, J. Y., Lee, B. R., Jin, L. H., Park, J. and Choi, S. Y. (2000) Transduction of Cu,Zn-superoxide dismutase mediated by an HIV-1 Tat protein basic domain into mammalian cells. *FEBS Lett.* **485**, 163-167.
39. Bae, J. Y., Lim, S. S., Kim, S. J., Choi, J. S., Park, J., Ju, S. M., Han, S. J., Kang, I. J. and Kang, Y. H. (2009) Bog blueberry anthocyanins alleviate photoaging in ultraviolet-B irradiation-induced human dermal fibroblasts. *Mol. Nutr. Food Res.* **53**, 726-738.
40. McCord, J. M. and Fridovich, I. (1969) Superoxide dismutase. *J. Biol. Chem.* **244**, 6049-6055.
41. Bradford, M. A. (1979) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
42. Lee, S. H., Kim, S. Y., Kim, D. W., Jang, S. H., Lim, S. S., Kwon, H. J., Kang, T. C., Won, M. H., Kang, I. J., Lee, K. S., Park, J., Eum, W. S. and Choi, S. Y. (2008) Active component of *Fatsia japonica* enhances the transduction efficiency of Tat-SOD fusion protein both *in vitro* and *in vivo*. *J. Microbiol. Biotechnol.* **18**, 1613-1619.