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Toxicity of Bilirubin and Detoxification by PEG–Bilirubin Oxidase Conjugate
A New Tactic for Treatment of Jaundice

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11.1. INTRODUCTION

Bilirubin, the end product of heme catabolism, is generally regarded as toxic and highly fatal in newborn infants and fulminant hepatitis. Bilirubin encephalopathy (kernicterus) is usually considered to be caused by the entry of circulating, free (albumin-unbound), unconjugated bilirubin into the cerebral tissue.1,2 Bilirubin conjugation with glucuronic acid takes place in the liver and the process is impaired in liver diseases. Our tactic for the treatment of jaundice is to decompose toxic bilirubin by the enzyme bilirubin oxidase, and for that purpose the enzyme is made into a polymer conjugate to improve its pharmacological properties.3

For clinical application, it is essential to confirm that these final products are less toxic than bilirubin, or are nontoxic to the major vital organs, and that they will be excreted rapidly from the bloodstream. In this study, we clarified the biological characteristics of the degradation products in vitro and in vivo. To demonstrate the toxic effects of bilirubin in a live animal model is complicated, so we examined bilirubin cytotoxicity and detoxification by bilirubin oxidase (BOX) in vitro.4
Toxicity of bilirubin to cellular activities has been investigated by many workers: it uncouples oxidative phosphorylation,\textsuperscript{5-7} it depresses both protein synthesis\textsuperscript{8,9} and DNA synthesis,\textsuperscript{10,11} it decreases ATP content,\textsuperscript{9,12} and it increases potassium leakage in various tissue culture cells.\textsuperscript{9} It is also recognized to be potentially immunosuppressive and immunotoxic by its direct cytotoxic effects on human lymphocytes,\textsuperscript{13} granulocytes,\textsuperscript{13} and macrophages.\textsuperscript{14} Furthermore, bilirubin-mediated hemolysis resulting from bilirubin–erythrocyte membrane interaction has been reported.\textsuperscript{15-17}

However, to our knowledge, no studies except ours\textsuperscript{3,4} have been conducted on the cytotoxicity of the degradation products that result from treatment of bilirubin with BOX. If no toxicity of these degradation products of bilirubin by BOX is found, then the therapeutic rationales using BOX become obvious. In this study, we examined the cytotoxicity of these breakdown products using the C 1300 neuroblastoma cell line of mouse. Further, urinary excretion of the degradation products was also elucidated in a rat model.

Previously, various methods were used to remove bilirubin, such as plasma exchange, steroid therapy, and phototherapy, but none has proven to have therapeutic value as a first choice regimen.

BOX used for the present approach is a highly specific enzyme for bilirubin (with $M_r$ 52,000), derived from the microorganism $Myrothecium verrucaria$ MT-1.\textsuperscript{18-21} Langer et al. reported a treatment for severe neonatal jaundice with an immobilized BOX column system.\textsuperscript{22,23} However, the column system has many inconveniences, such as physical confinement, clotting problems, and high incidence of infections.

In the past several years the use of chemical modification of proteins,\textsuperscript{24-29} particularly of enzymes, has grown rapidly. We and others have successfully synthesized a number of polymer-conjugated protein drugs, such as styrene-co-maleic acid conjugated neocarzinostatin (SMANCS),\textsuperscript{24,25} poly(ethylene glycol) (PEG)–L-asparaginase,\textsuperscript{26,27} PEG–superoxide dismutase,\textsuperscript{28} PEG–adenosine deaminase (ADA),\textsuperscript{29} and PEG–interleukin 2.\textsuperscript{30} We have now modified BOX with PEG to increase its plasma half-life, to diminish its immunogenicity, and to make it injectable and more effective.

### 11.2. EXPERIMENTAL PROCEDURE

#### 11.2.1. Bilirubin Oxidase (BOX) and Other Reagents

BOX, 3.5 U mg$^{-1}$, was obtained from Amano Pharmaceutical Co., Ltd., Nagoya, Japan. Crystalline bilirubin was obtained from Sigma Chemical Co., St. Louis, Mo. FDA (fluorescein diacetate) was obtained from Dojin Chemical Co., Ltd., Kumamoto, Japan. All other chemicals were from commercial sources. The bilirubin solution was prepared according to the methods described by Sugita et al.\textsuperscript{30} with a slight modification: bilirubin, used as unconjugated form, was dissolved in 0.1 M