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Research paper

Selection of single chain variable fragment (scFv) antibodies from a hyperimmunized phage display library for the detection of the antibiotic monensin

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ABSTRACT

Concerns over the occurrence of the veterinary antibiotic monensin (MW 671 Da) in animal food products and water have given rise to the need for a sensitive and rapid detection method. In this study, four monensin-specific single chain variable fragments (scFvs) were isolated from a hyperimmunized phage-displayed library originating from splenocytes of a mouse immunized with monensin conjugated to bovine serum albumin (BSA). The coding sequences of the scFvs were engineered in the order 5′-V_L-linker-V_H-3′, where the linker encodes for $Gly_{10}Ser_7Arg$. Three rounds of selection were performed against monensin conjugated to chicken ovalbumin (OVA) and keyhole limpet hemocyanin (KLH), alternately. In the third round of selection, two different strategies, which differed in the number of washes and the concentration of the coating conjugates, were used to select for specific binders to monensin. A total of 376 clones from round two and three were screened for their specific binding to monensin conjugates and positive clones were sequenced. It was found that 80% of clones from round three contained a stop codon. After removing the stop codon by site-directed mutagenesis, ten binders with different amino acid sequences were subcloned into the vector pMED2 for soluble expression in *Escherichia coli* HB2151. Four of these scFvs bound to free monensin as determined using competitive fluorescence polarization assays (C-FPs). IC_{50} values ranged from 0.031 and 231 μM. A cross-reactivity assay against salinomycin, lasalocid A, kanamycin and ampicillin revealed that the two best binders were highly specific to monensin.

1. Introduction

Monensin (MW 671 Da) belongs to the family of antibiotics known as carboxylic ionophore polyethers. The exterior make-up of this molecule is highly hydrophobic (Fig. 1), thus even salts of monensin have very low solubility in water and relatively high solubility in organic solvents such as acetone, methanol and chloroform (Pinkerton and Steinrauf, 1970). The antibacterial and antimalarial properties of monensin are attributed to its ability to transport Na^+, K^+ and protons across the lipid membrane of cells, which affects the net accumulation of protons, and results in a decrease of the intracellular pH (Pressman and Fahim, 1982). Monensin is regularly used as an additive in poultry feed for the prevention and treatment of coccidiosis caused by *Eimeria necatrix*, *E. tenella*, *E. acervulina*, *E. brunetti*, *E. mitis*, and *E. maxima* (Canadian Food Inspection Agency). It is also used in the beef and dairy industries to prevent coccidiosis caused by *E. bovis* and *E. zuernii* and as a growth promotant to increase the rate of weight gain, to minimize loss of body tone during lactation in dairy cows, and

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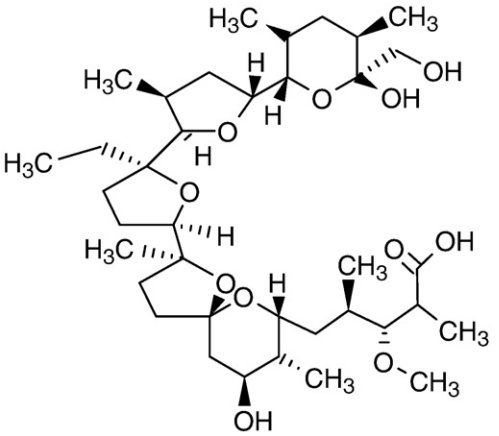

Fig. 1. Chemical structure of monensin (MW 671 Da).

to improve milk production in lactating dairy cows (Canadian Food Inspection Agency).

Various dosages of monensin can be toxic to a number of animals. In mice, the oral LD_{50} of monensin is 44 mg kg^{-1} (Pinkerton and Steinrauf, 1970). Monensin is also highly toxic to animals such as horses and dogs (Canadian Food Inspection Agency). Due to the frequent administration of monensin as a growth promotant in livestock, this antibiotic was detected in surface waters at seven different agricultural sites in Ontario (Hao et al., 2006). This has given rise to the need for a sensitive assay to detect this antibiotic in water samples to monitor and minimize the possibility of the environmental contamination with monensin.

There are two main challenges associated with producing high affinity antibodies and subsequently developing a sensitive assay against a hydrophobic hapten such as monensin. The first challenge lies in their lack of immunogenicity due to their small size. To overcome this problem, haptens have been covalently conjugated to larger molecules such as bovine serum albumin (BSA). However, immunization of an animal with a conjugated hapten could result in production of a heterogeneous pool of antibodies against the target hapten, conjugate, and carrier protein. Since haptens occupy a very small fraction of the immunogen, most of the B cells are triggered by the conjugate and carrier protein and only a few are activated by the haptens (Kramer, 2002). The second challenge with producing antibodies against hydrophobic haptens relies in the difficulties occurring during the conjugation reaction in which hydrophobic haptens may react with hydrophobic domains of carrier proteins. As a result of this reaction, haptens "hide" within the protein structure and thus are not exposed to B cells in the host animal or to antibodies during the selection process (Fasciglione et al., 1996).

To develop a sensitive and specific detection assay against monensin, we immunized four mice and two rabbits with either monensin-BSA or monensin-KLH (keyhole limpet hemocyanin) to determine the animal with the best immune response to monensin. The splenocytes of a mouse were utilized to construct a phage-displayed scFv library. Two different selection strategies were applied to isolate monensin-specific scFvs from this library by eliminating non-specific binders. We further describe the development of a fluorescence polarization (FP) assay to characterize the binding properties of the isolated scFv antibodies specific to monensin. Some of the problems associated with the isolation of scFvs against hydrophobic haptens are also discussed.

2. Methods and materials

2.1. Synthesis of monensin conjugates

Monensin was conjugated to three carrier proteins: BSA, OVA and KLH using the method described by Fleeker (1987). In brief, monensin (150 mg, 0.124 mM) was mixed with N-hydroxysuccinimide (50 mg, 0.124 mM) in 2 mL of methanol. To this solution, dicyclohexylcarbodiimide (39 mg, 0.124 mM) in 1 mL of methanol was added, mixed gently and incubated overnight at 22 °C in the dark. Either BSA (200 mg), OVA (ovalbumin; 200 mg) or KLH (200 mg) in 3 mL of borate buffer (0.1 M, pH 9.0) was added drop-wise to the vial with constant stirring. The reaction mixture was stirred for 2 h at 22 °C. The resulting conjugates were dialyzed against 1X phosphate-buffered saline (PBS; 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 per liter of water, pH 7.5) at 4 °C for 24 h.

2.2. Mice and rabbit immunization

Four eight-week-old Balb/c female mice and a pair of New Zealand white female rabbits were immunized with either monensin-BSA or monensin-KLH. Each conjugate was diluted in sterile 1X PBS and mixed with an equal volume of TiterMax Classic Adjuvant (Sigma-Aldrich Chemical Co., St. Louis, MO). For primary immunization, each animal was injected subcutaneously with 100 μg mL^{-1} of either conjugate. Two weeks after the primary injection, booster immunizations were administered at one week intervals. Each animal was injected with 50 μg mL^{-1} of a monensin conjugate mixed with an equal volume of Freund's incomplete adjuvant (*ca.* 100 μL; Sigma-Aldrich Chemical Co.). Sera (*ca.* 30 μL from mice and 500 μL from rabbits) were collected a week after each immunization and the immune response was monitored by ELISA. The last boost took place three days before sacrificing the animals.

The immune response was monitored against monensin-OVA and free monensin using indirect and competitive indirect (CI) ELISAs, respectively. For the indirect ELISA, the wells of the first row of microtitre plates were coated with 1:2000 (v/v) dilution of monensin-OVA (1 μg mL^{-1}) in 1X PBS, followed by a serial dilution (1:2 v/v across the plate) with 1X PBS. As a negative control, one row was coated with 5 μg mL^{-1} of OVA. The coated plates were incubated at 4 °C for 16 h. After washing the wells three times with 1X PBS, wells were blocked with 200 μL of 8% MPBS (8 g of skim milk in 100 mL of 1X PBS) for 2 h at room temperature. Wells were washed as described above and serum was diluted (1:50 and 1:100 v/v for mice and rabbits, respectively) in 1X PBS. After adding the diluted sera to the first column, a serial dilution (1:2 v/v across the plate) in 1X PBS was performed. The sera were allowed to bind to the conjugate by incubation for 1.5 h at room temperature. Wells were washed five times with 1X PBST (0.05% Tween 20 in 1XPBS) and incubated for 1 h with 100 μL of either polyclonal goat anti-mouse or goat anti-