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Covalent Binding of the Nitroso Metabolite of Sulfamethoxazole Is Important in Induction of Drug-Specific T cell Responses *in Vivo*

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Running title: Protein Binding and Immunogenicity of SMX-NO

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ABBREVIATIONS: IDHRs, immune-mediated drug hypersensitivity reactions; SMX, Sulfamethoxazole; SMX-NO, nitroso-sulfamethoxazole; MSA, mouse serum albumin; KLH, Keyhole Limpet Hemocyanin; s.c., subcutaneously; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant; ILN, inguinal lymph nodes; FCS, fetal calf serum; APCs, antigen-presenting cells, DTT, dithiothreitol; TCEP (Tris (2-Carboxyethyl)-Phosphine Hydrochloride); iodoacetamide (IACD)

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Abstract

Immune-mediated drug hypersensitivity reactions (IDHRs) represent a significant problem due to their unpredictable and severe nature, as well as the lack of understanding of the pathogenesis. Sulfamethoxazole (SMX), a widely used antibiotic, has been used as a model compound to investigate the underlying mechanism of IDHRs because it has been associated with a relatively high incidence of hypersensitivity. Previous studies by others showed that administration of SMX-NO, the reactive metabolite of SMX, to rats resulted in the generation of SMX-specific antibodies and *ex vivo* splenocyte proliferative responses, as well as haptenation of skin keratinocytes, circulating peripheral blood mononuclear cells and splenocytes. The objective of the present study was to further investigate SMX-NO-protein binding in relationship to its immunogenicity. In female DBA/1 mice treated with SMX-NO, varying degrees of SMX-NO-dependent T cell responses and SMX-NO-protein adduct formation were observed in the spleen, and inguinal, brachial and axillary lymph nodes. The data suggested a tissue-specific threshold of SMX-NO dosage that triggers the detection of adducts and immune response. Further, serum albumin and immunoglobulin were identified as protein targets for SMX-NO modification. It appeared that these adducts were formed in the blood, circulated to lymphoid tissues and initiated SMX-NO-dependent immune responses. Collectively, these data revealed a causal link between the deposition of SMX-NO-protein adducts in a lymphoid tissue and the induction of immune response in that tissue. Our findings also suggest that the immunogenicity of SMX-NO is determined by the immunogenic nature of the hapten, rather than special characteristics of the adducted protein.

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Immune-mediated drug hypersensitivity reactions (IDHRs), including allergic hepatitis, lupus, cutaneous reactions, and blood dyscrasias, account for approximately 6-10% of all adverse drug reactions (Adkinson, Jr. *et al.*, 2002). Although IDHRs are often referred to as rare (afflicting 1/100 to 1/100,000 patients), their unpredictable and serious nature makes them a significant problem in clinical practice and drug development. Sulfamethoxazole (SMX, chemical structure shown in Fig. 1) is a widely used antibiotic and it is particularly effective in the treatment of *Pneumocystic carinii* pneumonia in AIDS patients. Administration of SMX has been associated with a relatively high incidence of hypersensitivity reactions that manifest as fever and morbilliform cutaneous reactions (Cribb *et al.*, 1996). Hypersensitivity syndrome, manifested as fever, rash, eosinophilia, and hepatotoxicity, has also been reported in some patients receiving SMX (Berg and Daniel, 1987;Rieder *et al.*, 1989).

SMX hypersensitivity reactions generally occur 7 to 14 days after the initiation of therapy, which is reminiscent of a delayed occurrence of immune responses upon antigen stimulation. Immunohistochemical analyses revealed infiltrates of $CD4^+$ and $CD8^+$ T cells in the skin of patients afflicted with SMX-induced cutaneous reactions (Correia *et al.*, 1993;Miyauchi *et al.*, 1991). Further, it has been demonstrated that $CD4^+$ T cells isolated from SMX-hypersensitive patients proliferated in response to *in vitro* re-stimulation with both SMX and its reactive metabolite, 4-(nitroso)-N-(5-methyl-1,2-oxazol-3-yl)benzenesulfonamide (SMX-NO, chemical structure shown in Fig. 1) (Burkhart *et al.*, 2001;Schnyder *et al.*, 2000). Despite the above evidence for the involvement of the immune system in SMX hypersensitivity, the underlying mechanism accounting for the immune reaction remains to be elucidated. The hapten hypothesis predicts that a chemically reactive metabolite is generated and binds to endogenous proteins, and the drug-protein adduct serves as the antigen. In the case of SMX, its

oxidative metabolite, SMX-NO, has been identified as the protein-reactive intermediate (Naisbitt *et al.*, 2001;Manchanda *et al.*, 2002;Naisbitt *et al.*, 1999;Reilly *et al.*, 2000;Summan and Cribb, 2002). It has been demonstrated that administration of SMX-NO, but not SMX, resulted in cell surface haptenation of skin keratinocytes, circulating peripheral blood mononuclear cells, and splenocytes (Naisbitt *et al.*, 2001). Previous studies have also demonstrated that immunization of different species of animals, including mice, rats, and rabbits with SMX-NO, but not the parent drug, could induce specific T cell responses and anti-SMX antibody production (Naisbitt *et al.*, 2001;Gill *et al.*, 1997;Farrell *et al.*, 2003). These findings suggest that SMX-NO is more immunogenic than SMX. One possible explanation is that, compared with animals treated with SMX-NO directly, those treated with SMX may generate much less SMX-NO and SMX-NO-protein adducts. This is supported by the observation that SMX metabolism is compromised in mice (Farrell *et al.*, 2003).

In the present study, we set out to investigate whether the tissue distribution and the nature of drug-protein adducts are important factors in determining the immunogenicity of a drug, using SMX as a model compound. Our data revealed that the deposition of the SMX-NO-protein adducts in lymphoid tissues is important in the induction of immune responses *in vivo*.

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Materials and Methods

Animal Treatment. Female DBA/1 mice (7-10 week of age) were purchased from the Jackson Laboratory and kept in the Center for Laboratory Animal Care at the UCHSC for one week before treatments. For SMX treatment, the animals were intraperitoneally (i.p.) injected with SMX (50 mg/kg, dissolved in PBS containing 2% DMSO) or vehicle 4 times weekly for 3 wks. Four days after the last dose, mice were sacrificed, and splenocytes and lymph node cells isolated for *ex vivo* T cell proliferation assays. For SMX-NO treatment, mice were injected i.p. with SMX-NO (1, 2, 5, or 10 mg/kg, dissolved in PBS containing 2% DMSO) or vehicle 4 times weekly for 1 or 2 wks. Various times after the last dose, the mice were sacrificed, and splenocytes and lymph node cells isolated for proliferation assays. Some mice were sacrificed 6 hs after the last dose, and the liver, spleen, blood and lymph nodes collected for Western blot analyses to determine SMX-NO-protein adduct formation.

Immunization. SMX-NO-conjugates of mouse serum albumin (SMX-NO-MSA) and Keyhole Limpet Hemocyanin (SMX-NO-KLH) were synthesized by reacting SMX-NO (20 μg) directly with 6 mg of MSA or KLH dissolved in DMEM medium. Female DBA/1 mice were subcutaneously (s.c.) injected at the base of the tail with SMX-NO-MSA (50 μg protein/mouse) on days 0, 3, 7, 10, and 14. Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were included in the first and third immunization, respectively. One week after the last immunization, mice were sacrificed and inguinal lymph nodes removed for T cell proliferation assays.

Lymph node- and splenic-T cell proliferation assay. Female DBA/1 mice were treated with SMX, SMX-NO or immunized with SMX-NO-MSA as described above. Four

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days after the last dose of SMX-NO or SMX, or 1 wk after the last immunization with SMX-NO-MSA, mice were sacrificed, and the spleen and the inguinal, axillary and brachial lymph nodes removed. The cells were pooled from 3-5 mice, and single-cell suspensions prepared. The cells (1×10^6 cells/well) were stimulated with SMX-NO (10 μg/mL) and kept for 4 days in 96-well plates in DMEM containing 10% FCS (fetal calf serum). During the last 16 hs, the cells were pulsed with [^{3}H]-thymidine (0.5 μCi/well), and T cell proliferation determined by thymidine uptake. In some experiments, serum-free X-vivo medium (Lonza, Walkersville, MD) was used for the lymph node proliferation assays.

Albumin Immunoprecipitation. A goat anti-mouse albumin antibody (Bethyl Laboratories, Montgomery, Texas) was pre-incubated for 1 h with Protein A/G agarose (Santa Cruz, Santa Cruz, CA) at 4 °C in order to couple the antibody to the beads. Serum samples from mice treated with SMX-NO or vehicle were pre-cleared of immunoglobulin by an 1 h incubation with protein G agarose beads at 4 °C. The beads were pelleted by centrifugation at 15,000 rpm for 30 sec, and washed once in 50 mM sodium phosphate, pH 7.4. Subsequently, the beads were boiled in SDS loading buffer to remove immunoglobulin for SDS-PAGE analysis. The immunoglobulin-depleted supernatant was then subjected to immunoprecipitation overnight at 4 °C using the protein A/G agarose-coupled albumin antibody. Following overnight incubation, bound albumin was precipitated by centrifugation at 15,000 rpm for 30 sec. Beads were washed three times with sodium phosphate, pH 7.4, and bound albumin was removed by boiling beads in SDS buffer. Immunoglobulin, albumin, and supernatant fractions were analyzed by SDS-PAGE and immunoblotting using rabbit anti-SMX antisera.

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Western blot analysis. Female DBA/1 mice were treated with SMX-NO as described above. The animals were sacrificed 6 hs after the last dose, and blood, lymph nodes, spleen and liver collected. Serum and various tissue homogenates were prepared. Two μL of serum or 50 μg of tissue homogenate samples were diluted in Laemmli sample buffer (Bio-Rad) under reducing conditions, boiled for 5 min, and resolved on 12% polyacrylamide gels. After being transferred onto nitrocellulose membranes, nonspecific binding was blocked with 5% nonfat milk. The blots were probed with a rabbit polyclonal anti-SMX antibody (1:100, kindly provided by Dr. Craig Svenssen, Purdue University), and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2000, Chemicon International Inc). Protein signals were visualized using an ECL Plus Western Blotting Detection System (Amersham Bioscience), and the data were captured using a Storm 860 system (Molecular Dynamics, Sunnyvale, CA).

Results

Induction of Drug-Specific Immune Responses in Mice Treated with SMX-NO, but not SMX. Female DBA/1 mice were treated with either SMX (50 mg/kg) 4 times weekly for 3 wks or SMX-NO (1 mg/kg) 4 times weekly for 2 wks. Fours days after the last dose, the spleen, liver and various lymph nodes (inguinal, axillary and brachial) were removed, and T cell proliferation in response to *in vitro* re-stimulation with SMX-NO was determined. Treatment of mice with SMX-NO resulted in T cell responses in both the inguinal lymph nodes (ILN) and the spleen, with a stronger response in the ILN (Fig. 2A). No immune response was detected in the liver, or the axillary and brachial lymph nodes (data not shown). In contrast to those treated with SMX-NO, mice treated with SMX did not develop immune responses in any of the tissues described above (Fig. 2B and data not shown).

Interestingly, SMX-NO did not stimulate T cell proliferation in DMEM medium in the absence of FCS, whereas significant T cell proliferation was observed in DMEM medium containing 10% FCS (Fig. 3). To investigate whether FCS can be replaced by other proteins to cause T cell activation, we used X-vivo serum-free medium which contains a number of proteins, including human albumin, insulin and transferrin. The data showed that SMX-NO could stimulate T cell proliferation in X-vivo in the absence of FCS (Fig. 3). These results suggest that SMX-NO reacts with FCS or other proteins present in the culture media and forms SMX-NO-protein adducts, which in turn, serve as antigens to stimulate T cell activation. This finding argues against the possibility that SMX-NO stimulates T cell proliferation through direct modification of cell surface proteins on antigen-presenting cells (APCs).

To determine the time of T cell response occurrence, ILN cells were isolated at various time points after the last dose of SMX-NO. The results showed that the ILN cells obtained from mice 4 days, compared with 2 days or 2 hs, after the last dose of SMX-NO treatment, had greater responses to *in vitro* re-stimulation with SMX-NO (Fig. 4A). Further, we shortened the duration of the SMX-NO treatment (from 2 wks to 1 wk), varied the dose, and compared the levels of immune responses in different lymphoid tissues. SMX-NO-dependent T cell responses were observed in the ILN at all doses of SMX-NO treatments (2, 5, and 10 mg/kg), with the strongest response at 5 mg/kg (Fig. 4B). While T cell responses in the spleen were detected in mice treated with SMX-NO at 1 mg/kg for 2 wks (Fig. 2A), significant SMX-NO-dependent immune responses were observed in the spleen only after the mice were treated at a dose of 5 or 10 mg/kg, but not 2 mg/kg, for 1 wk (Fig. 4C). Similarly, T cell responses were only observed in the brachial and axillary lymph nodes at 5 and 10 mg/kg doses (Fig. 4D). No immune response was observed at any dose in the liver (data not shown).

Analysis of SMX-NO-Protein Adducts. To determine whether SMX-NO-dependent T cell response occurring in a lymphoid tissue was caused by the deposition of SMX-NO-protein adducts in that tissue, we performed immunoblot analyses using various lymphoid tissues obtained from mice treated with SMX-NO. The levels of SMX-NO-protein adducts in each tissue varied in relation to the dose of SMX-NO. High levels of adducts were observed in the ILN at all doses of SMX-NO treatments (2, 5, and 10 mg/kg, Fig. 5A). However, the level of SMX-NO-protein adduct formation was very low in the spleen and the brachial and axillary lymph nodes when mice were treated with 2 mg/kg of SMX-NO. Significant amounts of adducts were only detected in these tissues at 5 and 10 mg/kg doses (Figs. 5B and C). This tissue-specific threshold of SMX-NO

dosage that can trigger the detection of adducts is similar to the tissue-specific threshold that leads to immune responses, as shown in Fig.4.

Moreover, SMX-NO-protein adducts migrated at approximately 70 kDa, similar as those observed in lymphoid tissues, were also detected in the sera of mice treated with all doses of SMX-NO (Fig. 5D). Two additional minor protein adducts of lower molecular weight were also observed in the serum of SMX-NO-treated mice (Fig. 5D).

Identification of the SMX-NO-Protein Adducts Detected in Mice Treated with SMX-NO. The ILN homogenate was run on gels for Coomassie stains. The Coomassie bands in the region of 70 kDa were harvested and subjected to trypsin digestion and LC-MS/MS analysis in order to identify proteins present. These experiments showed that albumin was a major component of the 70 kDa band, but did not determine whether albumin was adducted by SMX-NO (data not shown). To determine whether albumin and/or other plasma proteins are targets for covalent adduction by SMX-NO *in vivo*, serum samples from treated mice and vehicle controls were subjected to depletion of immunoglobulin followed by albumin immunoprecipitation using an anti-albumin antibody. Subsequently, the SMX-NO-adducts were probed by immunoblot analysis using anti-SMX antisera. Both SMX-NO-immunoglobulin and SMX-NO-albumin adducts were detected in mice treated with SMX-NO, while adducts were not detected in vehicle controls (Fig. 6A and B). No adducts were detected in the serum fraction depleted of both immunoglobulin and albumin (data not shown). The results suggest that the SMX-NO-protein adducts formed in the blood after SMX-NO administration may reach the lymphoid tissues to cause SMX-NO-dependent T cell responses.

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Covalent Modification of Albumin by SMX-NO *in vitro*. SMX-NO has previously been reported to form covalent adducts with proteins, but these adducts have not been thoroughly characterized (Sanderson *et al.*, 2007). In order to determine whether SMX-NO could covalently modify albumin *in vitro*, native MSA (0.5 mg/mL) was reacted with SMX-NO (1 μg/ml). The reaction mixtures were subjected to Western blot analysis and SMX-NO-albumin adducts were detected (Fig. 7A). It has been reported that SMX-NO reacts with glutathione (Cribb *et al.*, 1991), suggesting its reactivity with free sulfhydryl. However, whether cysteine (Cys) residues of albumin are targets of adduction by SMX-NO has not been investigated. To examine this hypothesis, experiments were performed in which disulfide bonds were blocked and the protein was then incubated with SMX-NO. In order to block all Cys residues, MSA was first reduced by dithiothreitol (DTT), and the free Cys residues were then chemically blocked with iodoacetamide (IACD). Reduction of the disulfides prior to IACD alkylation was used in order to provide more stringent conditions than would be expected with simple alkylation of the native protein. The proteins with blocked Cys residues were then incubated with SMX-NO and immunoblotted for adducts. These experiments clearly showed that the adducts of albumin are localized on free Cys residue(s) of MSA, since no adducts were detected in the samples with pre-blocked Cys residues (Fig. 7A).

While human serum albumin contains only one nondisulfide cysteine residue, mouse serum albumin contains two (Cys58 and Cys603), and it was not clear from previous experiments whether one or both of these residues may be targets for SMX-NO adducts. Further attempts were made to confirm the presence of a Cys adduct. SMX-NO-protein adducted native albumin was digested using a combination of trypsin and chymotrypsin, and the resulting peptides were separated by liquid chromatography and subjected to tandem mass spectrometry (LC-MS/MS) using an Agilent 1100 series LC/ESI-MSD

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Trap. The MASCOT program was then used to search MS/MS ions from deconvoluted spectra in order to identify peptides corresponding to entries in the SwissProt database, and to identify adducts within peptides using entries in the UniMod database for the sulfinamide-, sulfenamide-, and semimercaptal-Cys adducts. Several independent experiments detected peptides, which are underlined in the sequence shown in Fig.7B. Although greater than 50% of the sequence of albumin was covered, the nondisulfide Cys residues were not detected.

Evaluation of the Immunogenicity of SMX-NO-MSA *in vivo*. SMX-NO-MSA and SMX-NO-KLH adducts were synthesized as described in the Method. Female DBA/1 mice were immunized with SMX-NO-MSA in conjunction with CFA or IFA. One week after the last immunization, the animals were sacrificed and ILN removed. SMX-NO-dependent T cell responses were evaluated by *ex vivo* re-stimulation of lymph node cells with SMX-NO and SMX-NO-KLH. The data demonstrated that the lymph node T cells proliferated upon *in vitro* re-stimulation with SMX-NO and SMX-NO-KLH, but not KLH (Fig. 8). The result suggests that SMX-NO-MSA represents an antigenic signal that induces SMX-NO-dependent T cell responses *in vivo*.

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Discussion

The hapten hypothesis proposes that metabolism of drugs to form chemically reactive metabolites and their covalent binding to endogenous proteins are necessary for the generation of antigens that elicit drug-specific immune responses. This hypothesis was recently challenged by the finding that T cells isolated from patients with SMX hypersensitivity reactions proliferated upon *in vitro* re-stimulation by the parent drug, independent of metabolite and covalent binding to proteins (Schnyder *et al.*, 2000;Mauri-Hellweg *et al.*, 1995;Schnyder *et al.*, 1997). Although the data are convincing, they do not exclude the possibility that the initiation of drug-specific immune response requires drug metabolism. Furthermore, a number of studies using various animal species demonstrated that SMX-NO is much more immunogenic than the parent drug (Naisbitt *et al.*, 2001;Gill *et al.*, 1997;Farrell *et al.*, 2003). Consistent with the evidence to support a necessary role of drug metabolism, our data demonstrated that SMX-NO, but not SMX, could induce T cell responses in lymphoid tissues of mice. The SMX-NO-protein adducts were also detected in these lymphoid tissues. The dose of SMX-NO treatment that caused greater amount of adduct deposition in a tissue also induced a greater SMX-NO-dependent T cell response in that tissue. These findings suggest that the potential for SMX to cause immune responses *in vivo* is determined by i) the amount of reactive metabolite formed, ii) the extent of covalent binding to proteins, and iii) exposure of the drug-protein adducts to the immune system.

It has been shown that SMX metabolism is compromised in mice (Farrell *et al.*, 2003), thus, compared with mice treated with SMX-NO directly, mice treated with SMX may generate less SMX-NO and SMX-NO-protein adducts. In the case of SMX treatment, the protein adducts formed within the target cells during metabolism need to be released

in order to be “seen” by the immune system; whereas after SMX-NO treatment, the protein adducts are likely formed in the circulation and are more readily “exposed” to the immune system. Previous studies comparing the immunogenicity of SMX and SMX-NO demonstrated T cell responses within the spleen of animals treated with SMX-NO (Naisbitt *et al.*, 2001;Farrell *et al.*, 2003). Additional lymphoid tissues were investigated in this study. SMX-NO-dependent T cell responses were observed in the spleen, as well as in the inguinal, brachial and axillary lymph nodes, although the degree of the response varied in each tissue in relation to the dose of SMX-NO treatment (Fig. 4). Further, the presence of FCS or other proteins in cell culture medium during *ex vivo* SMX-NO stimulation is necessary for the elicitation of T cell responses (Fig. 3). These data suggest that the *ex vivo* T cell response is elicited by hapten-protein conjugates generated in the medium and not via direct binding of SMX-NO to cell surface proteins. It is highly likely that the APCs may take up the SMX-NO-protein adducts and present the antigen to the T cells.

Furthermore, SMX-NO-protein adducts were detected in the serum, as well as in various lymphoid tissues, where SMX-NO-dependent T cell responses were induced (Fig. 5). There are three possible explanations for the detection of SMX-NO-protein adducts in tissues distant from the site of injection. First, SMX-NO may be stable enough to circulate in the periphery and reach these tissues, in which it forms protein adducts. Second, SMX-NO may non-covalently associate with serum albumin, which protects it from being degraded before reaching the lymphoid tissues. Third, SMX-NO may covalently bind to plasma proteins, and the adducts can subsequently travel to the lymphoid tissues. Our identification of immunoglobulin and albumin adducts of SMX-NO negated the possibility of non-covalent association. Given the high ratio of the blood flow to blood volume in mouse, it is possible that SMX-NO can reach significant

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concentrations at a site distant from where it is formed or administered. Therefore, SMX-NO-protein adducts may be formed in the lymphoid tissues locally, or the SMX-NO-protein adducts were formed in the circulation, and distributed to the lymphoid tissues and initiate immune responses. Furthermore, our data revealed a threshold of SMX-NO dosage above which SMX-NO-protein adducts could be detected, and this threshold varied for different lymphoid tissues (Fig. 5). These data indicated a causal link between the deposition of SMX-NO-protein adducts in a lymphoid tissue and the induction of immune response in that tissue.

It has been proposed that the nature of the protein that is covalently modified by the reactive metabolite of a drug may be important in determining the immunogenicity of the drug-protein adduct. However, our data demonstrated that i) immunoglobulin and albumin were targets for SMX-NO binding in the serum (Fig. 6), ii) in the absence of these proteins in x-vivo serum-free medium, SMX-NO still induced T cell proliferation (Fig. 3), and iii) SMX-NO-MSA administration could elicit SMX-NO-dependent T cell response (Fig. 8). These findings suggest that protein covalent modification by SMX-NO is not selective. The fact that albumin and immunoglobulin were the primary targets is most likely due to their abundance in the serum, rather than the particular characteristics of the proteins. The above results suggest that the immunogenicity of SMX-NO is dependent on the hapten itself rather than a special feature of the adducted protein.

Our *in vitro* experiments confirmed that SMX-NO could covalently modify mouse albumin, and the data revealed the involvement of one or both free cysteine residues (Fig. 7A). Previous work with human serum albumin has shown that Cys34 is highly reactive with electrophiles (Stewart *et al.*, 2005;Beck *et al.*, 2004), and it is probable that the corresponding Cys residue in MSA is the primary target of SMX-NO. However, we

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were not able to rule out the possibility that the second free Cys residue may also be a target for SMX-NO adduction. This is because we could not detect all the peptides of MSA using the selected instrument settings of electrospray mass spectrometry, and those undetected peptides contain the two nondisulfide cysteine residues (Fig. 7B).

In summary, we demonstrated that multiple doses of SMX-NO, but not SMX, could induce drug-specific T cell reactions in mice. We also found that the SMX-NO-protein adducts formed in the circulation travel to the lymphoid tissues, and that the amount of adducts in a tissue determines the extent of immune responses in that tissue. Although there are limited human studies on SMX metabolism to determine where SMX-NO-protein adducts are formed and whether they deposit into lymphoid tissues, we speculate that a similar mechanism to what we found in mice apply to human. Further, the finding that SMX-NO binds to immunoglobulin and albumin in the serum suggested that the protein modification is not selective, and that a protein becomes a target simply because of its abundance in the tissue, rather than its particular characteristics. Collectively, our findings using SMX-NO provide an alternative hypothesis in evaluating the potential of a drug to cause immune responses *in vivo*. The hypothesis is that the propensity of a drug in causing immune reactions is dependent on i) the formation of reactive metabolites and drug-protein adducts, ii) the immunogenicity of the drug hapten rather than the adducted proteins, and iii) the “exposure” of the drug-protein adducts to the immune system. The insights gained from the present study could help develop strategies to evaluate the potential of drug candidates to induce IDHRs.

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Footnotes:

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Figure Legends

Figure 1. Chemical structures of SMX and SMX-NO.

Figure 2. SMX-NO-dependent T cell responses were detected in the ILN and spleen of mice treated with SMX-NO but not SMX. Female DBA/1 mice were injected i.p. with SMX-NO (1 mg/kg, dissolved in 2% DMSO, panel A) 4 times weekly for 2 wks or SMX (50 mg/kg, dissolved in 2% DMSO, panel B) 4 times weekly for 3 wks. Control mice were injected i.p. with vehicle. Four days after the last dose, splenocytes and ILN cells were isolated and pooled from 3 mice in each group. The cells were cultured (1 x 10^6 cells/well) in 96-well plate for 4 days in the presence of SMX-NO (10 μg/ml). During the last 16 hs, the cells were pulsed with [^{3}H] thymidine (0.5 μCi/well) and T cell proliferation measured as counts per minute (CPM). The CPMs of unstimulated splenocyte and ILN cells obtained from SMX-NO treated mice were 2346 ± 151 and 1557 ± 341, respectively. Results from three independent experiments were combined and the values represent mean ± SEM. *P value <0.05 compared with vehicle-treated mice.

Figure 3. Presence of FCS or other proteins in the culture media is necessary for *in vitro* re-stimulation of SMX-NO-dependent T cell responses. Female DBA/1 mice were injected i.p. with SMX-NO (1 mg/kg in 2 % DMSO) or vehicle 4 times weekly for 2 wks. Four days after the last dose, the mice were sacrificed, and the ILN cells isolated and pooled from 3 mice in each group. The cells were re-suspended in serum-free X-vivo medium, or in DMEM in the presence or absence of 10% FCS. T cell proliferation assays were performed as described above. The experiments were carried out in

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triplicate and the results represent mean ± SEM. The CPM of unstimulated ILN cells obtained from SMX-NO-treated mice was 1886 ± 614. *P value <0.05 compared with vehicle-treated mice.

Figure 4. Evaluation of SMX-NO-dependent T cell responses in various lymphoid tissues of mice treated with varying doses of SMX-NO. Panel A, female DBA/1 mice were injected i.p. with 1 mg/kg of SMX-NO (dissolved in 2% DMSO) or vehicle 4 times weekly for 2 wks (days 1, 2, 3, 4, 8, 9, 10, and 11). At various times (2 h, 2 days, or 4 days) after the last dose, the mice were sacrificed, and ILN cells isolated and pooled from 3 mice in each group. T cell proliferation upon re-stimulation with 10 μg/ml of SMX-NO was determined as described above. Panels B, C, and D, female DBA/1 mice were injected i.p. with vehicle or various doses of SMX-NO (2, 5, and 10 mg/kg dissolved in 2% DMSO) 4 times weekly for 1 wk (days 1, 2, 3, and 4). Four days after the last dose (day 8), mice were sacrificed, and cells were isolated from the ILN (B), spleen (C), and brachial and axillary lymph nodes (D), and pooled from 3 mice in each group. T cell proliferation upon re-stimulation with 10 μg/ml of SMX-NO was determined as described above. The experiments were carried out in triplicate and the results represent mean ± SEM. *P value <0.05 compared with vehicle-treated mice.

Figure 5. **Detection of SMX-NO-protein adducts in various tissues of mice treated with SMX-NO.** Female DBA/1 mice were injected i.p. with vehicle or various doses of SMX-NO (2, 5 and 10 mg/kg dissolved in 2 % DMSO) 4 times weekly for 1 wk. Six hours after the last dose, the mice were sacrificed to collect various tissues, including ILN (A), spleen (B), brachial and axillary lymph nodes (C), and serum (D). Two μL of serum or 50 μg of various tissue homogenate samples were diluted in Laemmli sample buffer and

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resolved on 12% polyacrylamide gels. After transfer onto nitrocellulose membranes, the blots were probed with a rabbit anti-SMX antisera (1:100 dilution). Molecular mass markers are indicated on the right.

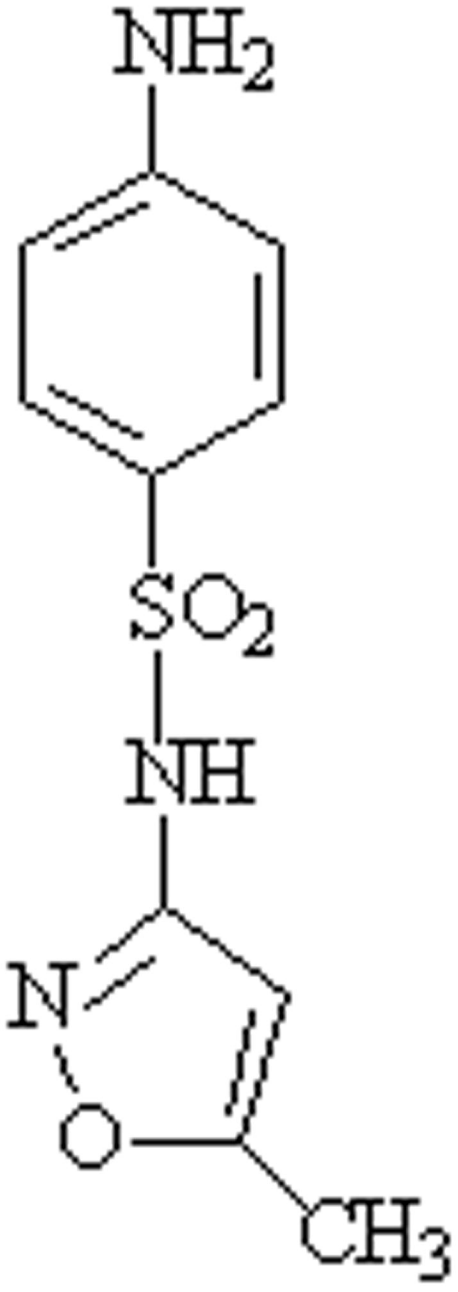
Figure 6. **Identification of albumin and immunoglobulin as targets for SMX-NO covalent modification in the sera of mice treated with SMX-NO.** Female DBA/1 mice were injected i.p. with SMX-NO (2 mg/kg in 2% DMSO) or vehicle 4 times weekly for 1 wk. Six hours after the last dose, mice were sacrificed, and serum prepared. Immunoglobulin and albumin were purified from the serum samples and probed for SMX-NO-adducts using rabbit anti-SMX antisera. The sample lanes were loaded with the immunoglobulin (A, 5 μL) or albumin (B, 5 μL) fractions of the serum samples obtained from mice treated with vehicle (lanes 1 – 3) or SMX-NO (lanes 4 – 6). Molecular mass markers (M) are indicated on the right.

Figure 7. **SMX-NO covalently binds to cysteine residues of albumin *in vitro***. (A) Immunoblot detection of SMX-NO adducts of MSA using a rabbit anti-SMX antisera in the following samples: native un-reacted MSA (lane 1), SMX-NO reacted with MSA in which the Cys residues were blocked by IACD after reduction by DTT (lane 2), and SMX-NO reacted with native MSA (lane 3). Molecular masses are indicated on the right. (B) Sequence coverage of SMX-NO-reacted native MSA. Underlined bold indicates peptides identified by LC-MS/MS analysis. Shaded italic bold indicates nondisulfide Cys residues.

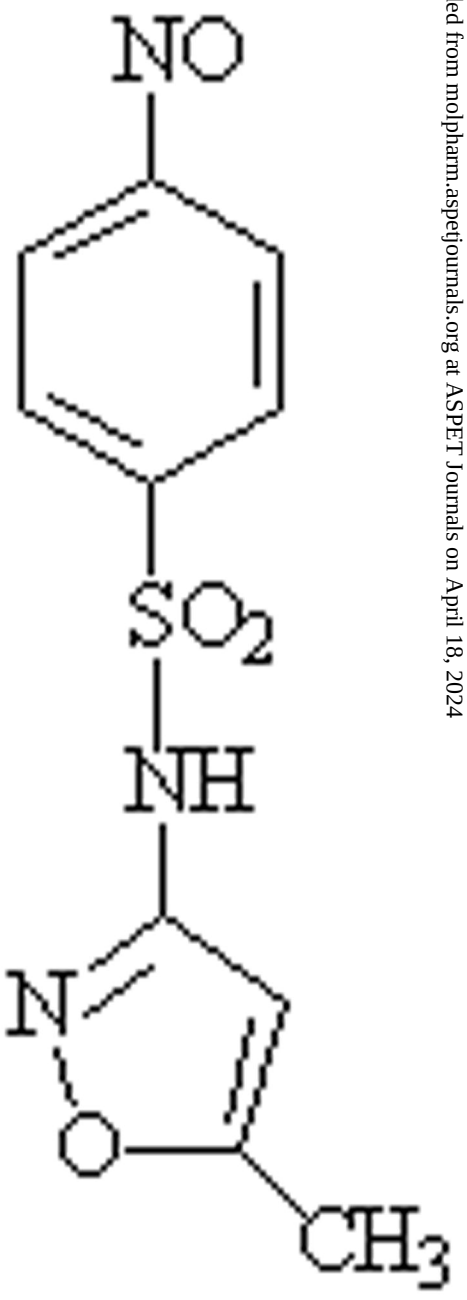
Figure 8. Immunization of mice with SMX-NO-MSA adducts induced SMX-NO-Dependent-specific T cell responses *in vivo*. Female DBA/1 mice were injected s.c.

at the base of the tail with SMX-NO-MSA (50 μg protein/mouse) on days 0, 3, 7, 10, and 14. CFA and IFA (50 μL) were included in the first and third immunization, respectively. One week after the last immunization, mice were sacrificed and the ILN cells isolated and pooled from 3 mice. Drug-specific T cell responses were evaluated by *in vitro* re-stimulation of ILN cells with SMX-NO (10 μg/ml, panel A), and 30 μg/ml of SMX-NO-KLH or KLH (panel B). The experiments were carried out in triplicate and the results represent mean ± SEM. *P value <0.05 compared with cell alone or KLH stimulation.

Fig.1



SMX



SMX-NO

Fig.2

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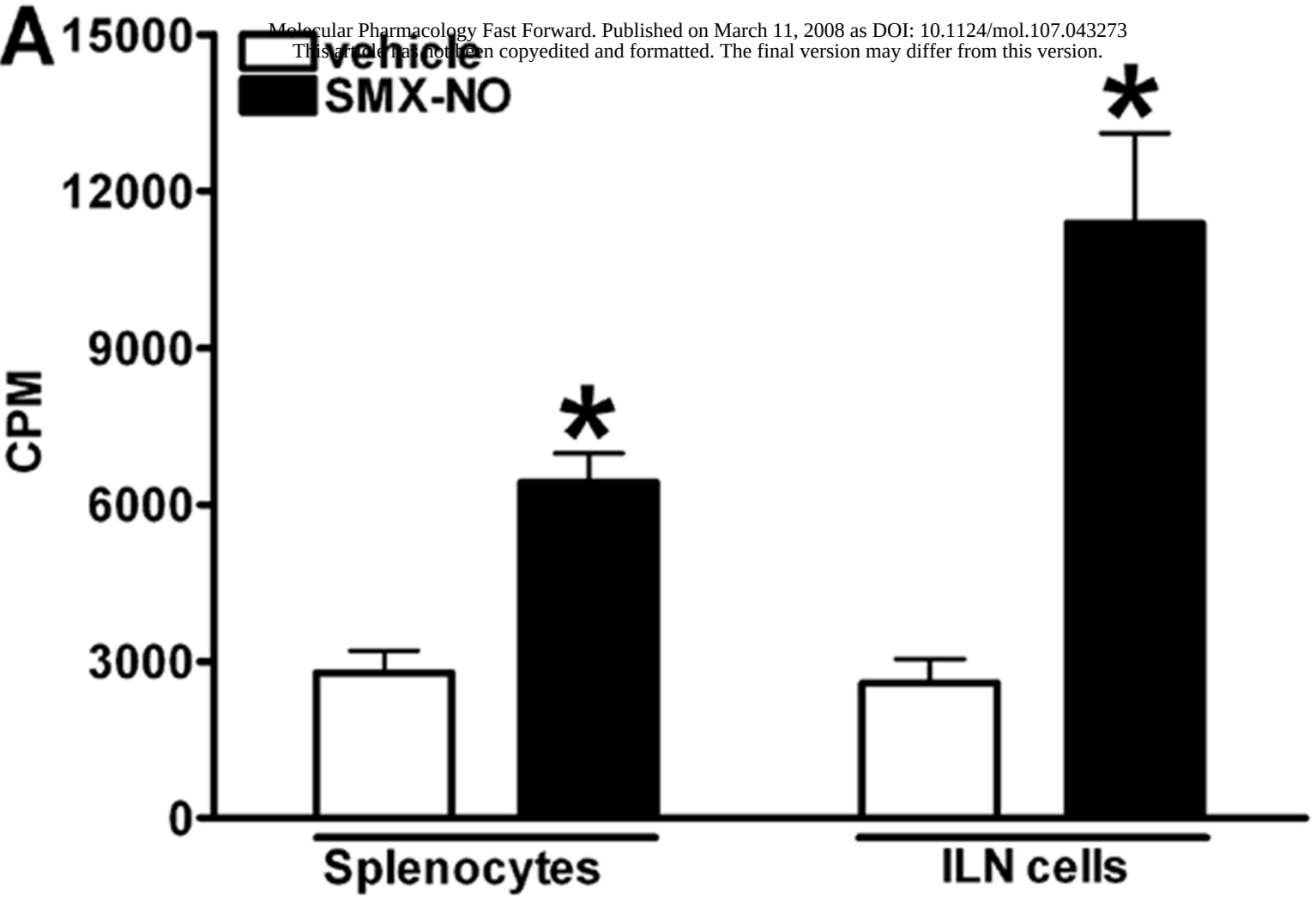


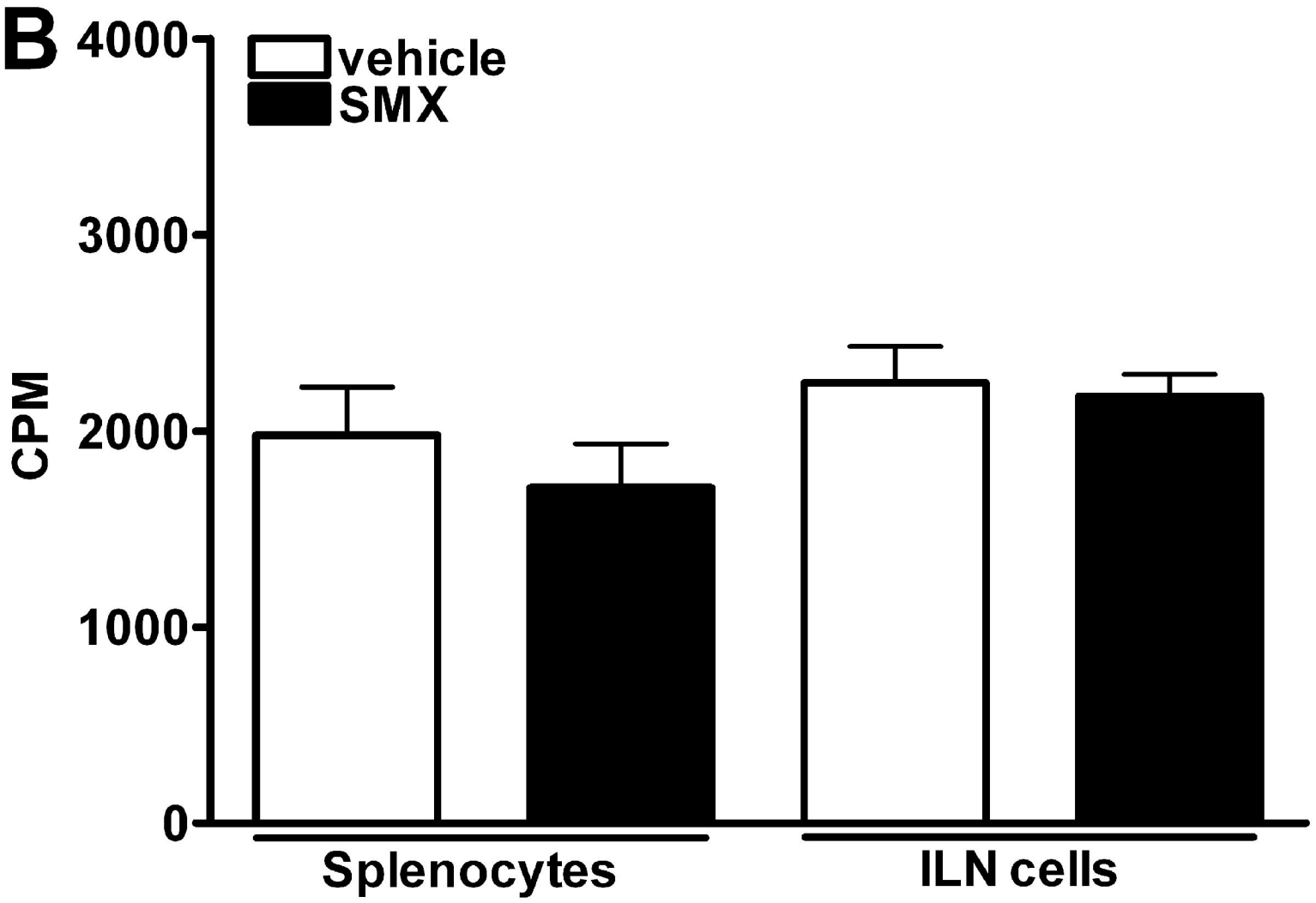


Fig.3

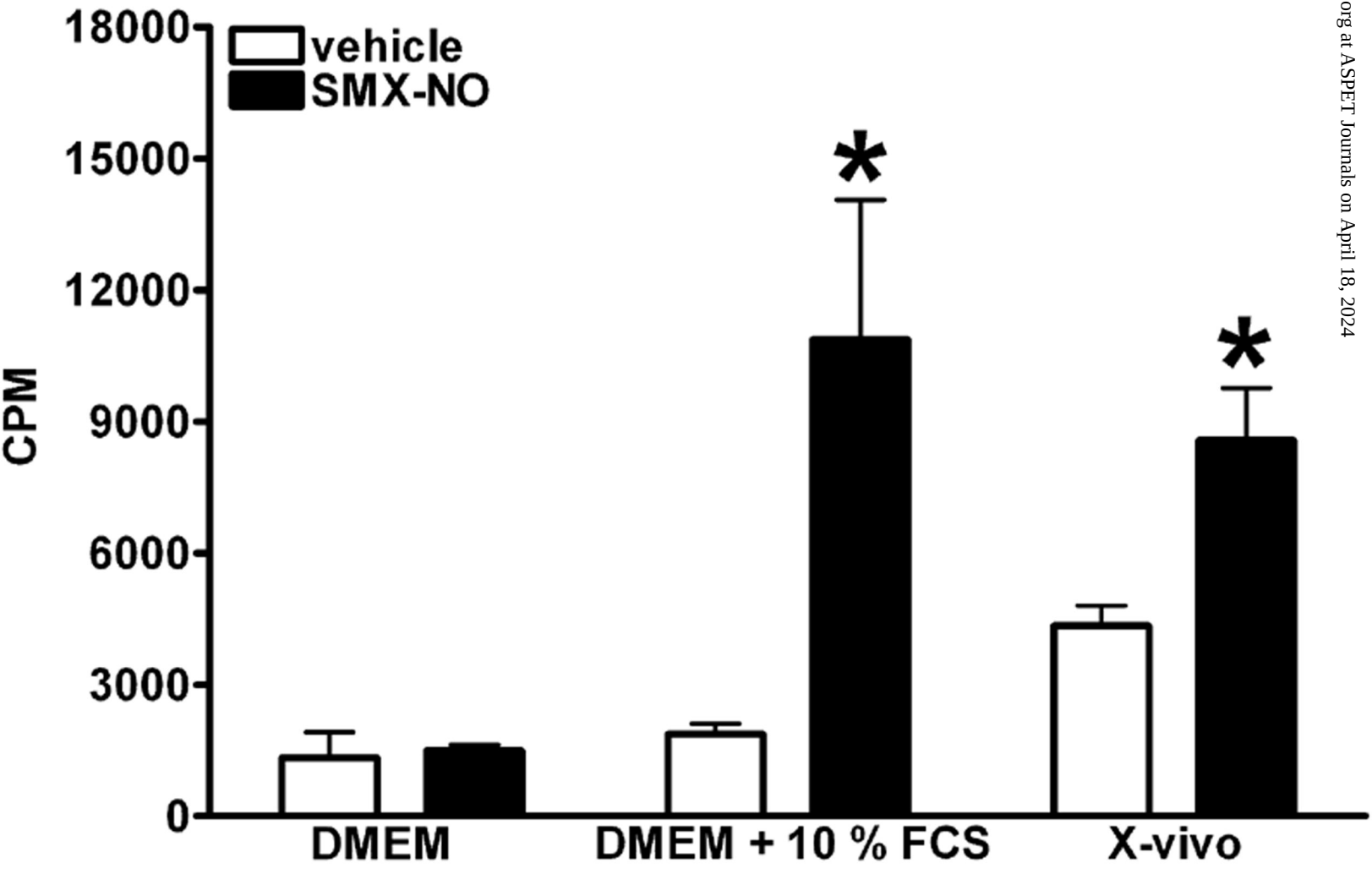
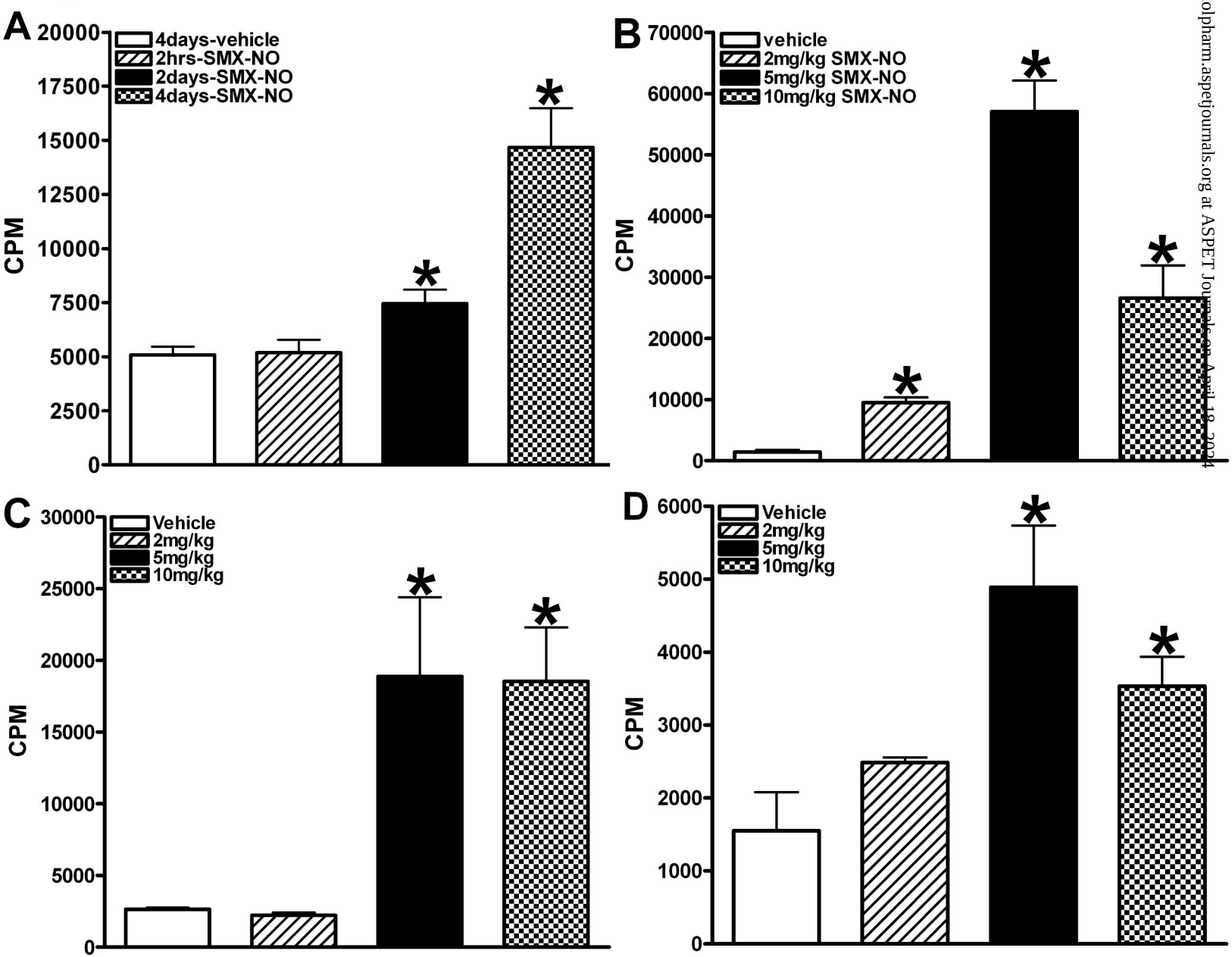


Fig.4



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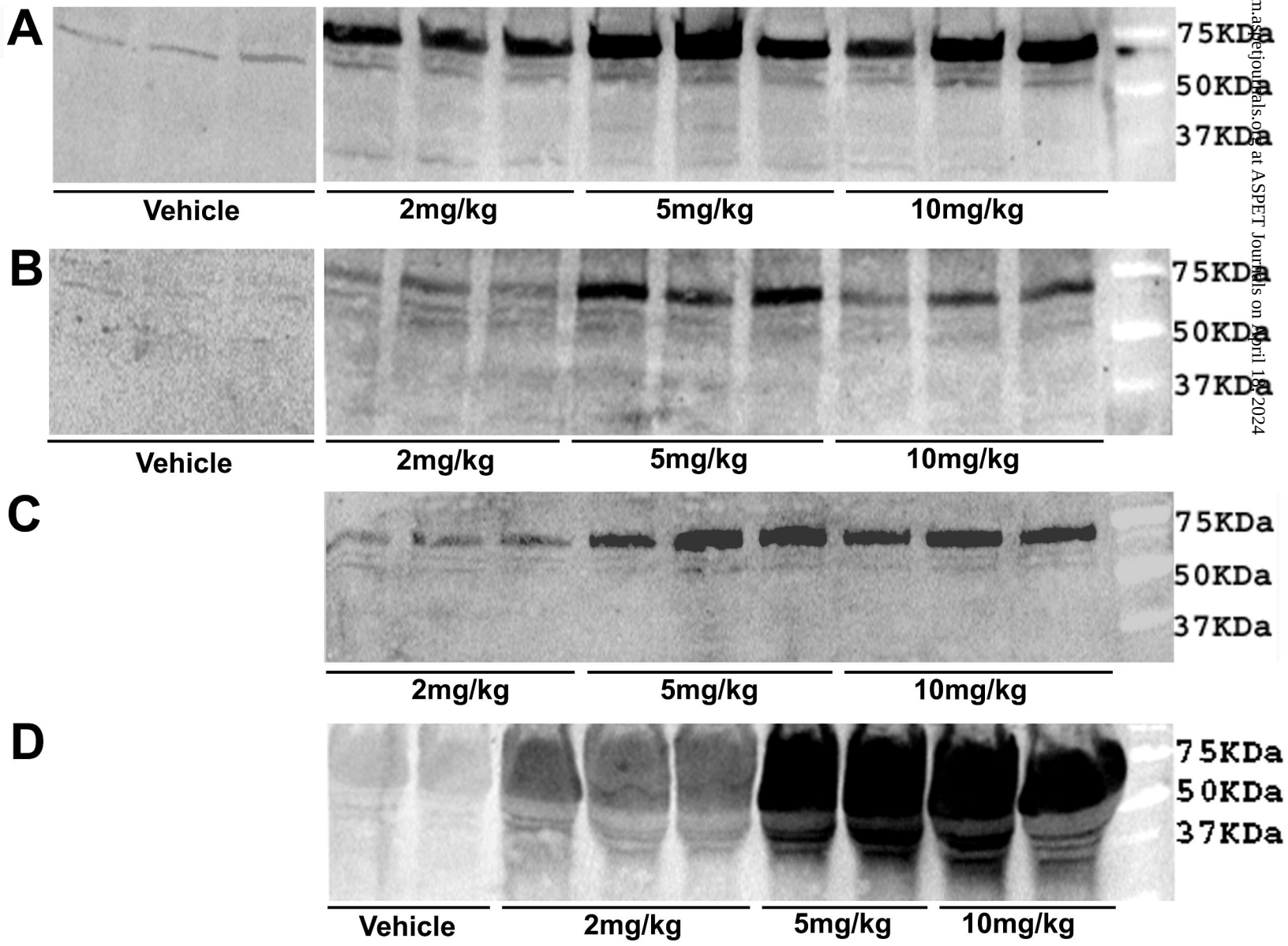


Fig.6

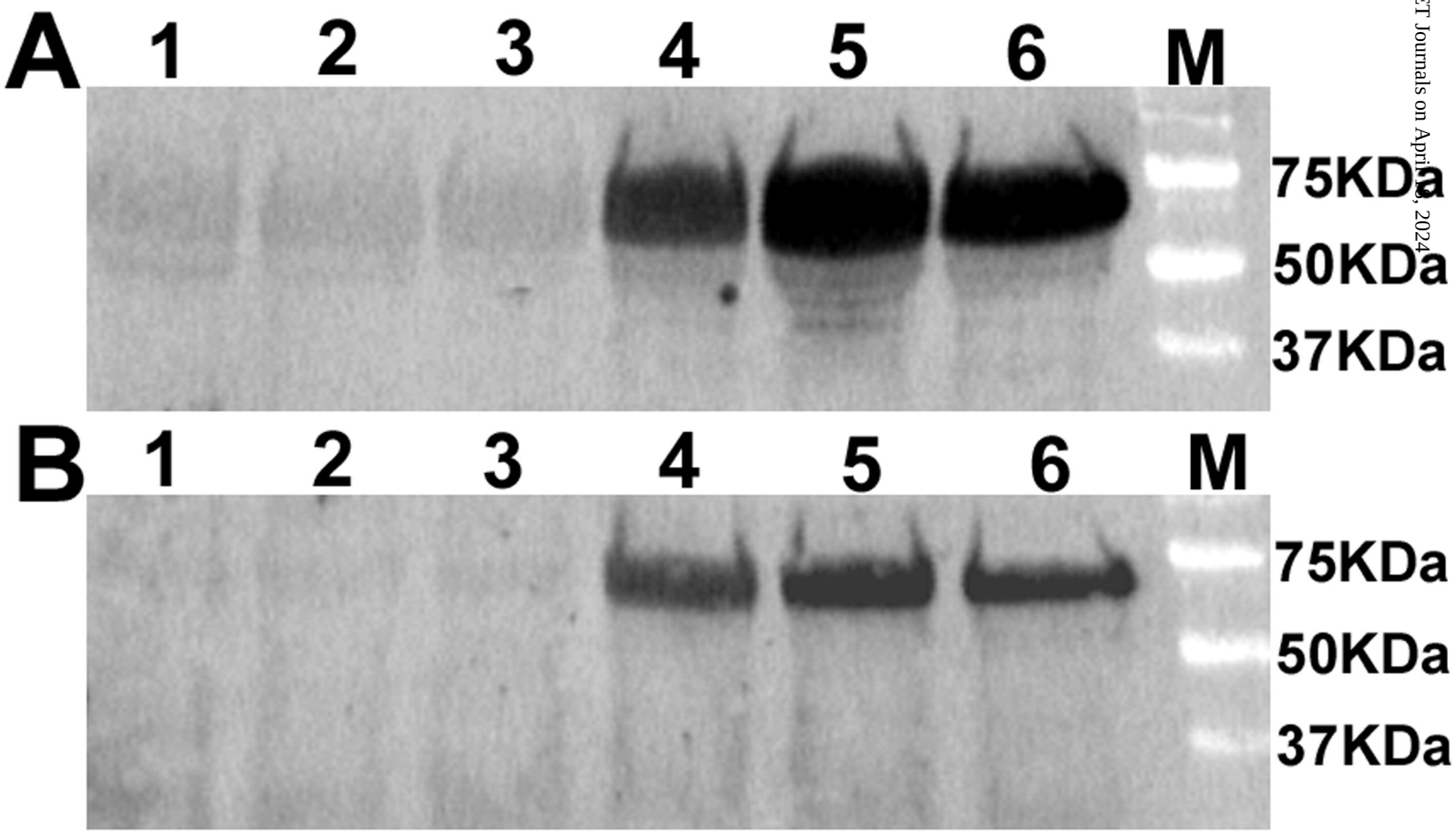
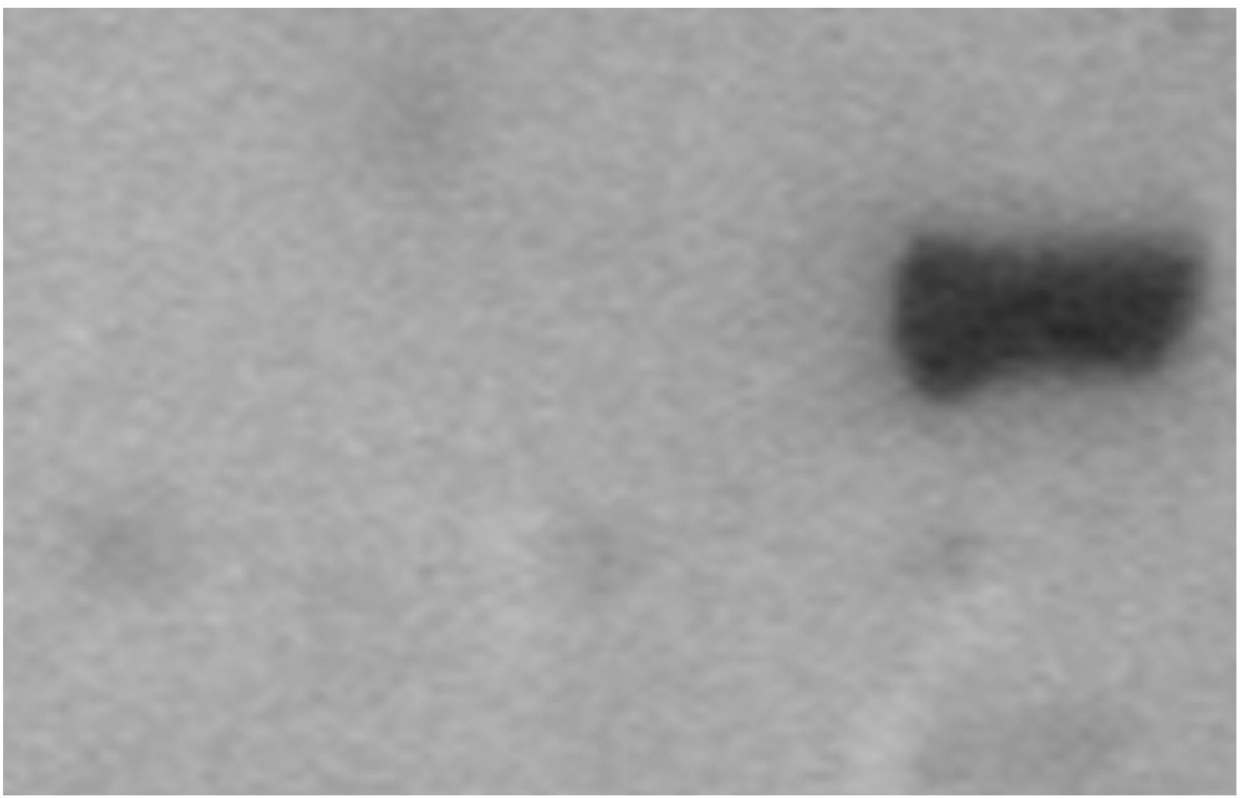


Fig.7

A



B

1 MKWVTFLLLL FVSGSAFSRG VFRREAHKSE IAHR**YNDLGE QHFK**GLVLIA

51 FSQYLQK***C***SY DEHAK**LVQEV TDFAKTCVAD ESAANCDK**SL HTLFGDK**LCA**

101 **IPNLRENYGE LADCCTKQEP ERNECF**LQHK **DDNPSLPPFE RPEAEAMCTS**

151 **FKENPTTF**MG HYLHEVARRH PY**FYAPELLY YAEQYNEILT QCCAEADKES**

201 **CLTPK**LDGVK EKALVSSVRQ RMKCSSMQKF GERAFKAWAV AR**LSQTFPNA**

251 **DFAEITKLAT DLTK**VNK**ECC HGDLLECADD R**AELAK**YMCE NQATISSKLQ**

301 **TCCDKPLLKK AHCLSEVEHD TMPADLPAIA ADFVEDQEVC K**NYAEAK**DVF**

351 **LGTFL**YEYSR **RHPDYSVSLL** LRLAKK**YEAT LEKCCAEANP PACYGTVLAE**

401 **FQPLVEEPK**N LVK**TNCDLYE** KLGEY**GFQNA ILVR**YTQK**AP QVSTPTLVEA**

451 **AR**NLGRVGTK **CCTLPEDQRL PCVEDYLSAI LNRVCLLHEK** TPVSEHVTK**C**

501 **CSGSLVERRP CFSALTVDET YVPK**EFKAET F**TFHSDICTL PEKEK**QIK**KQ**

551 **TALAELVK**HK PKATAEQLK**T VMDDFAQFL**D TCC**KAADKDT CFSTEGPNLV**

601 **TR*C***KDALA

Fig. 8

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