Allopurinol Increases Ear Swelling and Mortality in a Dinitrofluorobenzene-Induced Contact Hypersensitivity Mouse Model

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The immunomodulatory effects of allopurinol were investigated in a mouse contact hypersensitivity model. Allopurinol caused a time- and dose-dependent lethal effect in dinitrofluorobenzene (DNFB)-sensitized mice. Furthermore, allopurinol markedly increased ear swelling in the remaining mice. In contrast, TMX-67, a newly synthesized xanthine oxidase/xanthine dehydrogenase (XOD/XDH) inhibitor, had almost no effect on DNFB-sensitized mice. Allopurinol reduced both the spleen weight and white blood cell count in DNFB-sensitized mice without affecting the T cell subset of splenocytes. The production of interferon (IFN)- γ in the splenocytes of DNFB-sensitized mice was reduced by allopurinol administration. Death due to allopurinol was much lower in the non-sensitized mice than in the DNFB-sensitized mice. These findings indicate that allopurinol may interact with DNFB to enhance its toxicity and allopurinol might also modulate or enhance the inflammatory effect of DNFB. Also, DNFB may cause metabolic alterations *via* inflammation, leading to enhanced allopurinol toxicity.

Key words allopurinol; TMX-67; xanthine oxidase/xanthine dehydrogenase inhibitor; dinitrofluorobenzene; contact hypersensitivity; lethality

Hyperuricemia caused by a reduction in the renal excretion of uric acid, an excessive rate of uric acid production, or a combination of both, has been considered the most important risk factor for the onset of gout.^{1,2)} Agents such as uricosurics and xanthine oxidase/xanthine dehydrogenase (XOD/XDH) inhibitors, have been used to reduce serum uric acid levels. Currently the only commercially available XOD/XDH inhibitor is allopurinol, developed in the early 1960's.³⁾

Allopurinol is widely used and generally well-tolerated. However, when used in patients with renal insufficiency it may have life-threatening toxic effects, such as vasculitis, toxic epidermal necrolysis (TEN), eosinophilia, hepatitis, reduced renal function and bone marrow suppression, known as allopurinol hypersensitivity syndrome (AHS).^{4–7)} Allopurinol is metabolized to oxipurinol, an active metabolite,⁸⁾ which is excreted *via* the kidney into urine in the same manner as uric acid.^{9,10)} Oxipurinol tends to accumulate in patients with renal insufficiency. Although elevated oxipurinol levels seem to be a prerequisite for the occurrence of AHS,⁴⁾ the exact mechanism is unknown. Berken¹¹⁾ suggested that allopurinol-induced hypersensitivity may be due to impairment of suppressor T cell function resulting from inhibition of purine nucleoside phosphorylase (PNP).¹²⁾ However, there is no evidence that administering allopurinol to patients, actually impairs suppressor T cell function *via* inhibition of the PNP activity of T cells.

The aim of this study was to investigate the effect of allopurinol on immunological reactions using a mouse model of contact hypersensitivity induced by dinitrofluorobenzene (DNFB). This model is suitable for evaluating the effect of drugs on immunological reactions because a high sensitizing dose of DNFB can produce lower reactions by means of induction of suppressor T cells.¹³⁾ Furthermore, the effects of TMX-67 (2-(3-cyano-4-isobutoxyphenyl)-4-methyl-5-thiazole carboxylic acid), a newly synthesized non-nucleic acid structure XOD/XDH inhibitor (Fig. 1)^{14,15)} in this model were also investigated and compared with those of allopurinol.

MATERIALS AND METHODS

Experimental Animals Male BALB/c mice, 8 weeks of age (Charles River Japan, Kanagawa, Japan), were used for all experiments. The animals were kept in an air-conditioned room and given standard chow (CE-2, Clea Japan, Shizuoka, Japan) and water *ad libitum* for the duration of the study.

Materials The following materials were used: TMX-67 (synthesized by Teijin Ltd.), allopurinol (Sigma Chemicals Co., St. Louis, MO, U.S.A.), dinitrofluorobenzene (DNFB, Wako Pure Chemical Industries, Osaka, Japan), phycoerythrin (PE)-conjugated rat anti-mouse CD8a antibody (IgG2a), fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 antibody (IgG2a), PE-conjugated rat IgG2a κ , FITC-conjugated rat IgG2a κ , mouse recombinant interleukin (IL)-2, rat anti-mouse IL-2, biotin-conjugated anti-mouse IL-2, mouse recombinant interferon (IFN)- γ , rat anti-mouse IFN- γ , biotin-conjugated anti-mouse IFN- γ , mouse recombinant IL-4, rat anti-mouse IL-4, biotin-conjugated anti-mouse IL-4, mouse recombinant IL-10, rat anti-mouse IL-10, biotin-conjugated anti-mouse IL-10 (Pharmin-gen, San Diego, CA, U.S.A.), RPMI-1640 (GIBCO BRL, life technologies Inc., N.Y., U.S.A.), Phytohemagglutinin (PHA, Difco Laboratories, Detroit, MI, U.S.A.), avidin-biotin-horse-radish peroxidase (HRP) (ABC kit, VECTOR Laboratories, Burlingame, CA, U.S.A.) and tetramethylbenzidine (TMB, Kirkegaard & Perry Laboratories, MD, U.S.A.).

Sensitization with DNFB The abdomen of mice was

Fig. 1. Chemical Structures of Allopurinol (A) and TMX-67 (TEI-6720) (B)

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shaved with electric clippers and an electric razor on day -2. Mice were sensitized on days -1 and 0 by painting 100 μ l 0.5% DNFB, dissolved in ethanol, onto the shaved abdomen.

Contact Hypersensitivity Reaction Contact hypersensitivity reactions were induced by applying 10 μ l 0.3% DNFB dissolved in acetone and olive oil 4:1 (v/v) to both surfaces of one ear of sensitized mice on day 6. The other ear was painted with 10 μ l acetone and olive oil 4:1 (v/v) on both surfaces. Ear thickness was measured with a spring-loaded micrometer (Peacock G-1A, Ozaki MFG. Co., Ltd., Tokyo, Japan) before, and 24 and 48 h after, application of the challenge dose and ear swelling was calculated by the increase in ear thickness. The specific increment was calculated by subtracting the increase in the thickness of the ear painted with acetone and olive oil from that of the challenged ear. After the last measurement of ear thickness was made, mice were killed by an overdose of ether-anesthesia and their spleens were removed and weighed.

In another experiment, on day 6, blood samples were collected into a 1-ml plastic syringe with a 26G needle, filled with 1/10 volume of solution containing 3.8% EDTA-2Na by cardiac puncture under ether anesthesia. The white blood cell count was determined in a hematology analyzer (Celltac MEK-1200S, Nihon Kohden, Tokyo, Japan).

Preparation of Splenocytes On day 6, spleens were removed from mice treated with either ethanol-painting, DNFB-sensitization and MC-administration or DNFB-sensitization and 30 mg/kg/d allopurinol, and minced in Hank's balanced salt solution (HBSS, Whittaker Bioproducts, Inc., MD, U.S.A.). The cells were filtered through a Cell Strainer (FALCON, 70 μ m) and washed with HBSS. Lysis of erythrocytes in the suspension was performed by ammonium chloride in Tris HCl buffer. Then the splenocyte suspension was washed twice with HBSS.

Flow Cytometric Analyses A T cell suspension was prepared from the splenocyte suspension using a nylon fiber column (Wako Pure Chemical Industries, Osaka, Japan). The T cell suspension was adjusted to 1×10^7 /ml, and double-stained with PE-anti-CD8a antibody and FITC-anti-CD4 antibody. In brief, PE-anti-CD8a antibody and FITC-anti-CD4 antibody (1 μ g each) were added to 10^6 T cells, mixed and reacted at 4 $^{\circ}$ C for 45 min. The reaction mixture was washed with phosphate buffered saline (PBS) and then suspended in 1 ml PBS. As a negative control, a T cell suspension incubated with PE-rat IgG2a κ and FITC-rat IgG2a κ was prepared. Flow cytometric analysis was performed using an EPICS-CS flow cytometer (Coulter Electronics Inc., Hialeah, FL).

Cytokine Assays Splenocyte suspension was adjusted to 2×10^6 /ml in 1 ml RPMI-1640 containing 10% fetal calf serum, 100 μ g/ml streptomycin and 100 U/ml penicillin in 24-well microplates. The cells were stimulated with 1, 10 and 100 μ g/ml of PHA and cultured at 37 $^{\circ}$ C in a humid CO₂ incubator. Twenty-four, 48 and 72 h after stimulation, the supernatant was collected and IL-2, IFN- γ , IL-4 and IL-10 levels were measured by enzyme-linked immunosorbent assay (ELISA).

Treatment of Animals with Test Compounds TMX-67 (3, 10, 30 or 100 mg/kg/d) or allopurinol (3, 10, 30, 100 or 300 mg/kg/d) suspended in 0.5% methylcellulose (MC) solution, was orally administered, simultaneously with DNFB

Fig. 2. Effect of Allopurinol (A) or TMX-67 (B) on Survival Rate of DNFB-Sensitized Mice

Allopurinol (3, 10, 30 and 100 mg/kg/d), TMX-67 (3, 10, 30 and 100 mg/kg/d) or vehicle (0.5% MC) was given orally simultaneously with DNFB sensitization on days -1 and 0. Survival rate was recorded up to day 6.

sensitization, for 2 d. Control animals received only the dosing vehicle at a constant volume of 10 ml/kg.

Statistical Analyses For evaluation of the data, Dunnett's multiple range test was performed for differences in the effects of several doses of allopurinol or TMX-67 on the increment in ear thickness, spleen weight or changes in body weight. Student's *t*-test was performed to evaluate the differences in white blood cell counts of DNFB-sensitized mice between allopurinol- and vehicle-treated animals. The results are expressed as means $\pm$ S.E.M.

RESULTS

Contact Hypersensitivity Reactions A time- and dose-dependent reduction in survival rate was observed in sensitized mice given 30 mg/kg/d or more allopurinol and, by day 2, all the mice that received 100 mg/kg/d allopurinol were dead (Fig. 2A). In contrast, 9/10 mice given TMX-67 at a dose of 100 or less than 100 mg/kg/d (3, 10, 30 mg/kg/d) were still alive at day 6 (Fig. 2B).

On day 6, contact hypersensitivity reactions were induced in the remaining mice. The increase in the ear thickness of mice was markedly augmented by allopurinol (Fig. 3A). In contrast, TMX-67 did not affect the increase in ear thickness (Fig. 3B).

Allopurinol reduced the spleen weight of mice in a dose-

Fig. 3. Effect of Allopurinol or TMX-67 on the Increase in Ear Thickness in DNFB-Induced Contact Hypersensitivity Reactions

Allopurinol (3, 10, 30 and 100 mg/kg/d), TMX-67 (3, 10, 30 and 100 mg/kg/d) or vehicle (0.5% MC) was given orally simultaneously with DNFB sensitization on days -1 and 0. On day 6, the ear thickness of the surviving mice was measured before and 24 and 48 h after application of a challenge dose and the degree of swelling was obtained by calculating the increase in ear thickness. Data are calculated by subtracting the increase in the thickness of the ear painted with acetone and olive oil from that of the challenged ear and expressed as means $\pm$ S.E.M. * $p < 0.05$: statistically different from Control Group (Dunnett's multiple range test).

dependent manner (Fig. 4). A marked reduction in spleen weight was observed in mice given 30 mg/kg/d allopurinol. The change in body weight from day -1 of mice given 30 mg/kg/d allopurinol was also significantly reduced compared with that of control mice (-4.2 ± 0.6 g vs. -2.0 ± 0.3 g; $p < 0.01$). Mice given 30 mg/kg/d of TMX-67 also had a significant reduction in spleen weight, but not to the same extent as the allopurinol-treated mice. No further reduction in spleen weight was observed in mice given 100 mg/kg/d TMX-67 (Fig. 4). TMX-67 had no significant effect on the change in body weight from day -1 (data not shown). In addition, the number of white blood cells, including lymphocytes, in the peripheral blood of mice given 30 mg/kg/d of allopurinol was significantly reduced compared with that of control mice ($39 \pm 5 \times 10^5/\text{ml}$ vs. $52 \pm 3 \times 10^5/\text{ml}$; $p < 0.05$).

Flow Cytometric Analyses and Cytokine Assays We next examined the profile of T cells obtained from allopurinol-treated mice. T cell subsets of splenocytes were examined by flow cytometry on day 6. There was no difference in the ratio of CD4 single-positive cells and CD8 single-positive cells among the ethanol-painted mice (ethanol group), DNFB-sensitized and MC-treated mice (control group) and DNFB-sensitized mice given 30 mg/kg/d allopurinol (allopurinol group) (Table 1).

Fig. 4. Effect of Allopurinol or TMX-67 on Spleen Weight in DNFB-Induced Contact Hypersensitivity Reactions

Allopurinol (3, 10, 30 and 100 mg/kg/d), TMX-67 (3, 10, 30 and 100 mg/kg/day) or vehicle (0.5% MC) was given orally simultaneously with DNFB sensitization on days -1 and 0. After the last measurement of ear thickness, as described in the legend to Fig. 3, mice were killed by an overdose of ether-anesthesia, and spleens were removed and weighed. Data are calculated as mg/g body weight and expressed as means $\pm$ S.E.M. ** $p < 0.01$: statistically different from Control Group (Dunnett's multiple range test).

Table 1. Effect of Allopurinol on T Cell Subsets in DNFB-Sensitized Mice

Group	T cell subset (%)	
	CD4 ⁺ CD8 ⁻	CD8 ⁺ CD4 ⁻
Ethanol	57 $\pm$ 3	16 $\pm$ 4
Control	48 $\pm$ 9	16 $\pm$ 9
Allopurinol	53 $\pm$ 3	22 $\pm$ 5

Allopurinol (30 mg/kg/d) or vehicle (0.5% MC) was given orally simultaneously with DNFB sensitization on days -1 and 0. On day 6, T cell subsets of splenocytes were examined by flowcytometric analysis. Results represent the means $\pm$ S.E.M. of 3 experiments.

urinol group) (Table 1).

We next investigate whether allopurinol inhibited the production of suppressive Th2-type cytokines, such as IL-4 and IL-10, or enhanced the production of Th1-type cytokines, such as IL-2 and IFN- γ . Under the experimental conditions used, no production of Th2-type cytokines following PHA stimulation was observed (data not shown). IL-2, a Th1-type cytokine, was produced following PHA stimulation for 48 h, as shown in Fig. 5A, in a concentration-dependent manner. Although IL-2 production was increased in the splenocytes of DNFB-sensitized mice (control group) compared with those of ethanol-painted mice (ethanol group), allopurinol (allopurinol group) did not enhance IL-2 production. A study of 100 $\mu\text{g}/\text{ml}$ PHA stimulation kinetics revealed that increased IL-2 production by DNFB-sensitization was not affected by allopurinol-administration at any culture period (Fig. 5B). Another Th1-type cytokine, IFN- γ was also produced following PHA stimulation for 48 h and maximal production of IFN- γ was observed following stimulation with 10 $\mu\text{g}/\text{ml}$ PHA (Fig. 6A). Marked enhancement of IFN- γ production was observed in the splenocytes of DNFB-sensitized mice (control group). However, in the splenocytes of mice that were DNFB-sensitized and given 30 mg/kg/d allopurinol (allopurinol group), IFN- γ production was reduced

Fig. 5. Effect of Allopurinol on IL-2 Production in Splenocyte of DNFB-Sensitized Mice *ex Vivo*

Allopurinol (30 mg/kg/d) or vehicle (0.5% MC) was given orally simultaneously with DNFB sensitization on days -1 and 0. On day 6, spleens were removed and splenocyte suspensions prepared. The cells were stimulated with PHA and cultured in a humid CO₂ incubator at 37°C. After stimulation, the supernatant was collected and IL-2 levels were measured by ELISA. (A): The cells were stimulated with 1, 10 and 100 μg/ml PHA for 48 h. (B): The cells were stimulated with 100 μg/ml PHA for 24, 48 and 72 h.

Fig. 6. Effect of Allopurinol on IFN-γ Production in Splenocytes of DNFB-Sensitized Mice *ex Vivo*

Allopurinol (30 mg/kg/d) or vehicle (0.5% MC) was given orally simultaneously with DNFB sensitization on days -1 and 0. On day 6, spleens were removed and splenocyte suspensions prepared. The cells were stimulated with PHA and cultured in a humid CO₂ incubator at 37°C. After stimulation, the supernatant was collected and IFN-γ levels were measured by ELISA. (A): The cells were stimulated with 1, 10 and 100 μg/ml PHA for 48 h. (B): The cells were stimulated with 10 μg/ml PHA for 24, 48 and 72 h.

rather than enhanced, compared with the Control Group. These observations were also confirmed at 24 and 72 h after stimulation with 10 μg/ml PHA (Fig. 6B).

Survival Rate We observed a time- and dose-dependent reduction in survival rate in DNFB-sensitized mice given 30 mg/kg/d or more of allopurinol. This lethal effect seemed to be enhanced by DNFB. We determined the survival rate after the administration of allopurinol in non-sensitized mice painted with ethanol, instead of DNFB, and compared it with that in DNFB-sensitized mice. As shown in Fig. 7, the survival rate was much higher in non-sensitized mice than in DNFB-sensitized mice.

DISCUSSION

The present study clearly showed that allopurinol markedly augmented the increases in the ear thickness of DNFB-sensitized mice, suggesting enhancement of the contact hypersensitivity reaction. A newly synthesized XOD/XDH inhibitor, TMX-67, had almost no effect on DNFB-sensitized

Fig. 7. Effect of Allopurinol on Survival Rate of DNFB-Sensitized or Ethanol-Painted Mice

Allopurinol (30, 100 and 300 mg/kg/day) was given orally simultaneously with DNFB sensitization or ethanol painting on days -1 and 0. Survival rate was recorded up to day 6.

mice. In previous studies, we demonstrated that TMX-67 was 3–10 times more potent than allopurinol in rodents.¹⁴⁾ Therefore, the effects of allopurinol observed in the present study are not related to XOD/XDH inhibitory activity.

Allopurinol and oxipurinol have been shown to affect purine and pyrimidine biosynthesis in addition to XOD/XDH, because of their structural similarity to purine and pyrimidine bases. Both allopurinol and oxipurinol are known to undergo conversion to their corresponding nucleotides *in vivo*,¹⁶⁾ possibly *via* some phosphoribosyltransferases. Both allopurinol and oxipurinol inhibit the purine metabolic enzyme PNP¹²⁾ and Berken¹¹⁾ suggested that the inhibition of PNP by oxipurinol may cause an impairment of suppressor T cell function leading to AHS. However, in a mouse study, Nishida and co-workers¹⁷⁾ showed that the nucleoside metabolite of allopurinol suppressed, rather than enhanced, delayed-type hypersensitivity as a result of inhibition of PNP.

In addition to enhancement of ear swelling, the spleen weight and the white blood cell count of mice given 30 mg/kg/d allopurinol were reduced compared with control mice. These results seem conflicting. However, cyclophosphamide, which causes a reduction in white blood cell count, is known to enhance contact hypersensitivity reactions¹⁸⁾ possibly *via* inhibition of suppressor T cells.¹⁹⁾ Therefore, one may assume that the present conflicting results indicate that suppressor T cells might also be specifically reduced by oral allopurinol, as suggested by Berken.¹¹⁾

Therefore, we examined the profiles of T cells obtained from allopurinol-treated mice. However, the flow cytometric analyses in this study showed little difference in the ratio of CD4⁺ and CD8⁺ cells in the spleen T cells due to allopurinol, indicating that allopurinol does not reduce the CD8⁺ suppressor T cell count in mice. Expecting induction of suppressor T cells, we selected a high sensitizing dose of DNFB,¹³⁾ however no differences were observed between the ethanol-painted mice and DNFB-sensitized mice as far as the ratio of CD4⁺ and CD8⁺ T cells was concerned. In the DNFB-induced contact hypersensitivity model, both CD4⁺ and CD8⁺ T cells were reported to act as effector cells, and some CD4⁺ T cells are suppressive.²⁰⁾ In addition, dinitrothiocyanobenzene (DNTB) is reported to induce tolerance in the DNFB-induced contact hypersensitivity reaction *via* a shift in the balance of Th1 and Th2 cytokines in favor of the latter.²¹⁾ A recent report also showed that both CD4⁺ and CD8⁺ T cells could be divided into Th1- and Th2-type cells.²²⁾ These reports suggest that, in the DNFB-induced contact hypersensitivity model, enhancement of ear swelling by allopurinol might be explained by a balance of Th1 and Th2 cytokines, not by any alteration in T cell subsets.

Therefore, we next investigated the Th1 and Th2 cytokine pattern of splenocytes to clarify whether allopurinol inhibited suppressive Th2-type cytokines, such as IL-4 and IL-10, or enhanced Th1-type cytokines, such as IL-2 and IFN- γ . At least under the present experimental conditions, no Th2-type cytokines were produced even in the splenocytes of DNFB-sensitized mice. In contrast, Th1-type cytokine production was increased in the splenocytes of DNFB-sensitized mice compared with those of ethanol-painted mice. However, allopurinol did not increase the production of IL-2, but reduced the production of IFN- γ . These results also fail to explain the enhancement of ear swelling produced by allopuri-

nol. Reduction in the production of Th1-type cytokines by allopurinol should actually lead to immunosuppression, instead of immunostimulation. In fact, several reports have shown that allopurinol and its metabolites have immunosuppressive properties. Allopurinol inhibits blastogenic responses of human lymphocytes *in vitro*,²³⁾ and a nucleoside metabolite of allopurinol suppresses delayed-type hypersensitivity in mice *in vivo*.¹⁷⁾ From these reports and our own findings, augmentation of the increase in ear thickness produced by allopurinol may be due to some unknown mechanism(s) other than immunostimulation. In the present study, a time- and dose-dependent reduction in survival rate was observed in sensitized mice given 30 mg/kg/d or more allopurinol and by day 2, all the mice given 100 mg/kg/d allopurinol were dead. The LD₅₀ value of orally administered allopurinol in ddY mice is reported to be over 1000 mg/kg.²⁴⁾ Although strain differences should be taken into account, the toxicity of allopurinol in DNFB-sensitized mice in the present study is much higher than that reported by Isa and co-worker's.

Therefore, we next compared the survival rate of DNFB-sensitized mice with that of non-sensitized mice after administration of allopurinol, and found that the survival rate of DNFB-sensitized mice was clearly lower than that of non-sensitized mice. This result indicates that there is some biological interaction between allopurinol (or its metabolites) and DNFB, resulting in the increased ear swelling, lymphocyte damage and death rate. DNFB is known to possess both irritant and allergenic properties, causing inflammation, epidermal hyperplasia, induction of immediate early gene *c-fos* and *c-jun* expression and an increase in ornithine decarboxylase activity.²⁵⁾ Recent reports show that allopurinol enhances radiation lethality in mice²⁶⁾ and that the toxicity of allopurinol is enhanced in spontaneously hypertensive rats.²⁷⁾ These reports suggest that allopurinol may modulate or enhance the inflammatory reactions of DNFB, and/or that DNFB may cause metabolic changes *via* inflammation, leading to the enhanced toxicity of allopurinol. The pathogenesis of AHS involves some immunologic processes. Deposition of IgG has been found on the glomerular basement membranes of the kidneys of a patient dying from AHS.²⁸⁾ Furthermore, Utsinger and Yount²⁹⁾ found granular deposition of IgM at the dermal-epidermal junction of a patient with AHS. Some adverse effects of allopurinol may be mediated by allergic reactions to allopurinol or its metabolite(s).^{30–33)} However, the exact mechanism responsible for the development of AHS is unknown. Present studies suggest that AHS might also be caused by biological interactions between allopurinol (or its metabolite(s)) and some metabolic alterations. In patients with renal insufficiency, lower doses of allopurinol, based on individual creatinine clearance, are used to reduce the occurrence of AHS.⁴⁾ However, Hosoya and co-workers³⁴⁾ have shown that reduced doses of allopurinol are often insufficient to produce the desired hypouricemic effect. This suggests that, in patients with renal insufficiency, the balance between the efficacy and toxicity of oxipurinol may be altered in favour of the latter. If so, it is possible that in patients with renal insufficiency, renal failure might cause some metabolic alterations, as interaction with oxipurinol or its metabolite and lead to AHS, as in the present study. However, further investigation of the interaction between allopurinol and DNFB is necessary.

In conclusion, although we could not clarify the mechanism(s) of the enhanced ear swelling produced by allopurinol, we showed enhanced toxicity of allopurinol in combination with DNFB. In contrast, a newly synthesized XOD/XDH inhibitor, TMX-67, had little effect on DNFB-sensitized mice.

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