Cumhuriyet Sci. J., 46(2) (2025) 264-267
DOI: https://doi.org/10.17776/csj.1485123

Cumhuriyet Science Journal

| csj.cumhuriyet.edu.tr | Founded: 2002 ISSN: 2587-2680 e-ISSN: 2587-246X Publisher: Sivas Cumhuriyet University

Non-Competitive Inhibition of Xanthine Oxidase by N-Nitrosomorpholine: An In Vitro Study

Deniz BAKIR [1,a], Serkan KAPANCIK [2,b,*]

[1] *Biochemistry Laboratory, Medical Faculty Hospital, Sivas Cumhuriyet University, Sivas, Türkiye*

[2] *Department of Biochemistry, Faculty of Medicine, Sivas Cumhuriyet University, Sivas ,Türkiye*

**Corresponding author*

Research Article

History
Received: 16/05/2024
Accepted: 13/05/2025

ABSTRACT

Nitrosamines can be formed from nitrate. Nitrate is not actually toxic to mammals but can be reduced to nitrite. Nitrite, on the other hand, reacts with amino groups to form the carcinogenic N-nitroso compound. N-nitrosomorpholine (NMOR) is a carcinogenic compound that is included in the nitrosamine class and is the most common type of nitrosamines. Xanthine oxidase (XO) is an enzyme that catalyzes the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid in purine metabolism. Excessive production and/or inadequate excretion of uric acid causes hyperuricemia. This condition is associated with kidney and cardiovascular diseases, especially gout. In order to prevent hyperuricemia and therefore gout, suppressing the activity of the XO enzyme is one of the main targets. In our study, the possible inhibitory effects of NMOR on the activity of the XO enzyme were investigated by spectrophotometric method. XO activity was evaluated in the presence of different concentrations of NMOR and analyzed using Lineweaver-Burk plot. The Km value was determined as 0.55 mM and the Vmax value was determined as 2.45 U/ml. Finally, it was determined that the XO enzyme was inhibited non-competitively by NMOR. Inhibition of XO enzyme with the help of NMOR may lead to functional deficiencies by disrupting the pathway in which purines are metabolized, as well as mediating the control of the production of uric acid formed in purine metabolism. More advanced methods and in vivo studies are needed to better understand the effects of NMOR on the organism and XO enzyme.

Keywords: Xanthine Oxidase, Nitrosamine, Nitrosomorpholine, Non-competitive inhibition, Purine metabolism.

[a] *denizbakir1314@hotmail.com* *https://orcid.org/0000-0002-9255-3301* [b] *serkankapancik@gmail.com* *https://orcid.org/0000-0003-3019-4275*

Introduction

Nitrosamines can be formed from nitrate. Nitrate is not actually toxic to mammals but can be reduced to nitrite. Nitrite reacts with amino groups to form the carcinogenic N-nitroso compound. Nitrosamines are known as amine derivatives containing the R1R2N-N=O functional group. Nitrosamine has been reported to be carcinogenic to humans by the International Institute for Research on Cancer [1,2]. In order to maintain the freshness of meat products and preserve their color and flavor, nitrate and nitrite salts are added to them during production. Thus, although the microbial load is reduced and the shelf life of meat products is improved due to its effects on Salmonella, Clostridium botulinum, and mesophilic bacteria, this process also brings some risks. Especially nitrites and nitrates added to meat products form N-Nitrosamine compounds as a result of reactions involving nitrosating agents. Therefore, consumption of meat products produced with the addition of additives such as nitrite and nitrate can result in the formation of N-nitrosamine through nitrosating agents in the presence of stomach acid. Nitrosamine formation causes the risk of diseases such as cancer and negatively affects public health [3,4].

NMOR is a carcinogenic compound that is included in the nitrosamine class and is the most common type of nitrosamines. It is the nitrosamine with the fastest formation reaction. It can be formed as a result of the nitrosation reaction between morpholine and $NaNO_2$. The in-vivo synthesis of NMOR in the human body is quite high compared to its direct intake from the environment [5]. NMOR has been detected in sewage systems where wastewater is discharged, in workplace air, in tobacco products, and in drugs [6-9]. When NMOR was given to rats with drinking water, it was determined that it mediated hepatocarcinogenic formation, and also mediated changes in the expression of pyruvate kinase L and M2 isoenzymes, and the expression of pyruvate kinase L shifted towards the expression of pyruvate kinase M2 [10]. It was determined that morphological changes occurred in the bronchial epithelial cells of Syrian golden hamsters exposed to NMOR and that NMOR exposure mediated lung tumor formation in these animals [11]. However, in another study examining the development of oncocytic cells in the thyroid, adrenal gland, pituitary gland, pancreas, testis and femur bone of mice exposed to NMOR, oncocytic cells were detected in different endocrine tissues in more than half of the mice exposed to NMOR. Although the endocrine tissues in which these cells were observed varied from mouse to mouse, the thyroid, adrenal gland, pituitary gland and pancreas were

determined as the endocrine tissues in which these cells were observed [12].

The XO enzyme is an enzyme that catalyzes the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid. It was first purified from cow's milk. It is found in nature in a highly protected form. This is because organisms in nature need to metabolize purines. However, the enzyme is found as a dehydrogenase in most organisms except mammals. Excessive production and/or inadequate excretion of uric acid causes hyperuricemia, which is a significant health problem worldwide. Hyperuricemia is caused by excess uric acid in the blood as a result of the destruction of nucleic acids such as adenine and guanine. This can cause kidney function losses, metabolic disorders, gouty arthritis and cardiovascular diseases. Since the uricase enzyme, which is found in many organisms and allows uric acid to be broken down into allantoin, is not found in humans, the XO enzyme, which is responsible for the synthesis of uric acid, is the main target for the treatment of hyperuricemia and gout caused by hyperuricemia. Although some compounds, especially allopurinol, topiroxostat and febuxostat, which have XO inhibition, have been discovered and used for therapeutic purposes in medicine, these compounds have undesirable side effects. Therefore, studies on new compounds that mediate XO inhibition are useful for human health. These inhibition studies of the enzyme are important for the determination of effective compounds or extracts that have the potential to be used in the treatment of diseases caused by the activity of XO [13-15].

In our study, we aimed to look at the effects of NMOR, which is one of the nitroso compounds we are exposed to through different factors in our environment, on purine metabolism. In line with this purpose, we examined the effect of NMOR on the activation of the XO enzyme in vitro.

Materials and Methods

XO Activity Determination

The basis of this method is the formation of uric acid from xanthine by the enzyme xanthine oxidase and the measurement of the formed uric acid spectrophotometrically at a wavelength of 293 nm. Xanthine oxidase activity is calculated from the amount of uric acid formed per unit time by the formula. 1 µmol of urate formed per minute at pH 7.5 and 25°C was determined as one unit activity [16].

NMOR Interaction with XO

In order to find the in vitro effect of NMOR on XO activity, enzyme activities were determined against 2, 1, 0.5 and 0.3 mM substrate concentrations. NMOR was added to 4 different substrate concentrations as an inhibitor at 0, 20, 40 and 60 mM concentrations and enzyme activity was determined. Vmax and Km values of XO enzyme were calculated by drawing Lineweaver-Burk plot.

Results

Michaelis-Menten Plot was drawn according to the data obtained in vitro by using 4 different concentrations of XO enzyme substrate (2, 1, 0.5, 0.3 mM) (Figure 1) (Table 1).

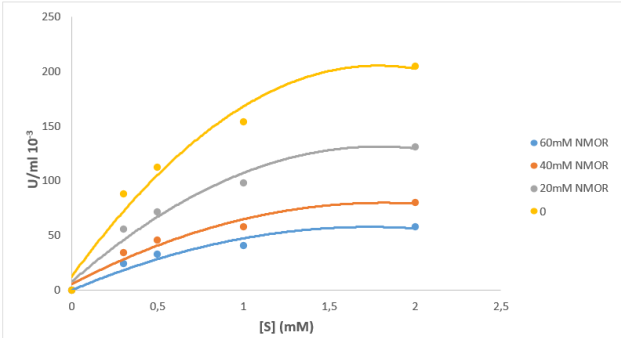

Figure 1. Michaelis-Menten Plot on inhibition of XO by NMOR

Lineweaver-Burk plot was drawn according to the data obtained in vitro by using 4 different concentrations of XO enzyme substrate (2, 1, 0.5, 0.3 mM). Vmax and Km values were calculated with the help of Lineweaver-Burk plot (Figures 2) (Table 2).

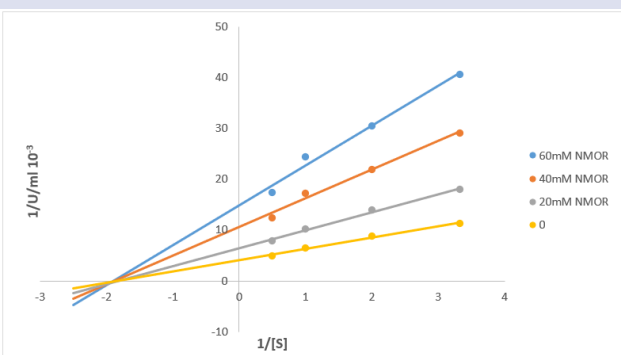

Figure 2. Lineveaver-Burke plot on inhibition of XO by NMOR

Table 2. Effects of NMOR on XO enzyme

	Inhibitor-free	20 mM NMOR	40 mM NMOR	60 mM NMOR
Vmax(U/ml).10^{-4}	2,45	1,59	0,95	0,67
Km(mM)	0,55	0,57	0,55	0,53

According to the data obtained from the Lineweaver-Burk curve, when NMOR inhibitor was not added to the reaction medium, Km of XO enzyme was determined as 0.55 mM and Vmax was determined as 2.45 U/ml 10^{-4}. When 20 mM NMOR inhibitor is added, the Km of the XO enzyme is 0.57 mM, Vmax is 1.59 U/ml 10^{-4}. When 40 mM NMOR inhibitor is added, the Km of the XO enzyme is 0.55 mM, Vmax is 0.95 U/ml 10^{-4}. When 60 mM NMOR inhibitor is added, the Km of the XO enzyme is 0.53 mM, Vmax is 0.67 U/ml 10^{-4} (Figure 2) (Table 2).

When the apparent Km value (Km value in the presence of inhibitor) and Vmax values of the enzyme were determined for each NMOR concentration with the help of these curves, a remarkable decrease was detected in the Vmax values of the enzyme with increasing NMOR concentration, as seen in Figure 2 and Table 2. In conclusion, non-competitive inhibition was confirmed as the Vmax values decreased while the Km values remained relatively unchanged.

Discussion

NMOR is a nitrosamine with carcinogenic effects. In particular, it plays a role in the formation of hepatocellular carcinomas [17]. It is known that nitrosamines inhibit the activity of many enzymes involved in the regulation of metabolism [18]. However, the literature study investigating the effects of NMOR on metabolism-regulating enzymes is quite limited.

It is known that the XO enzyme is responsible for purine destruction. Hyperuricemia, which occurs as a result of an increase in the activity of the enzyme, causes neurodegenerative diseases, especially gout, as well as chronic diseases such as cardiovascular diseases, diabetes, and persistent wounds. Therefore, XO enzyme is the main target for eliminating hyperuricemia. Inhibitors of this enzyme are currently used in the clinic for the treatment of diseases. However, these inhibitors have serious side effects that can be fatal. Therefore, research on XO inhibitors that cause minimal toxicity in the organism and have high inhibitory activity is important [19,20]. Therefore, in our study, due to the limited literature on the roles of NMOR on enzymes involved in metabolism; in order to contribute to the potential inhibitor studies of XO enzyme, the in vitro effect of NMOR on XO enzyme was investigated. In this study, XO was interacted with NMOR in the reaction medium containing different concentrations of xanthine substrate. The activities found were graphed with Lineweaver-Burk. In the graph, it was seen that NMOR inhibited XO non-competitively. It was determined that the enzyme interacting with NMOR lost its activity. The reason for this may be that NMOR mediated the suppression of its activity by binding to the enzyme protein when released into the medium.

In non-competitive inhibition, the inhibitor interacts with the enzyme to prevent product formation, whether the substrate molecule is bound or not. The inhibitor binds to a region separate from the substrate. It changes the conformation of the enzyme. The enzyme and the inhibitor are not similar in structure. There is no competition in enzyme binding. Since the EIS complex is degraded more slowly than the ES complex, the product yield and enzyme speed slow down. In non-competitive inhibition, Vmax values decrease while Km values remain relatively unchanged [21]. As a result of our study, since Vmax values decrease while Km values remain relatively unchanged, it was concluded that XO enzyme inhibition via NMOR is non-competitive inhibition in vitro. The in vitro results obtained from this study may indicate that NMOR can also inhibit purine degradation via XO enzyme in vivo. Thus, we can say that NMOR can prevent hyperuricemia in the body and the emergence of patients with hyperuricemia. However, in order to speak more definitively about the in vivo effects of NMOR on the XO enzyme, in vivo studies are needed on this subject.

Conclusions

We can say that NMOR has a non-competitive inhibitory effect on the purine metabolism enzyme XO in in vitro conditions, and that in this respect, NMOR may mediate the suppression of uric acid synthesis resulting from purine destruction in the body, and thus the hyperuricemia state. However, in vivo studies are needed to be sure about this issue.

Conflicts of interest

There are no conflicts of interest in this work.

Acknowledgement

There is no acknowledgement.

References

[1] Swann P.F., The toxicology of nitrate, nitrite and n-nitroso compounds, *Journal of the Science of Food and Agriculture*, 26 (11) (1975) 1761-1770.

[2] Ozbay S., Sireli U.T., Filazi A., Nitrosamines, their chemistries and effects on health, *Int. J. Sci. Technol. Res*., 5 (4) (2019) 124-133.

[3] Deveci G., Tek N.A., N-Nitrosamines: a potential hazard in processed meat products. *Journal of the Science of Food and Agriculture*, 104 (5) (2024) 2551-2560.

[4] Xie Y., Geng Y., Yao J., Ji J., Chen F., Xiao J., ... & Ma L., N-nitrosamines in processed meats: Exposure, formation and mitigation strategies. *Journal of Agriculture and Food Research*, *13* (2023) 100645.

[5] Li Y., Hecht S.S., Metabolic activation and DNA interactions of carcinogenic N-nitrosamines to which humans are commonly exposed, *International journal of molecular sciences,* 23 (9) (2022) 4559.

[6] Zhao B., Zhou J., Nakada N., Ihara M., Liu Y., Wong Y.J., ... & Tanaka H., COVID-19 impacts on characterization of N-nitrosamines and their precursors during transport in sewer systems, *Water Research*, 279 (2025) 123439.

[7] Meng X.J., Liu X.D., Zhang X.M., Hu Y., Guo Q.F.,. Determination of 8 N-nitrosamines in the workplace air by GC-MS/MS method, *Chinese journal of industrial hygiene and occupational diseases*, 42 (8) (2024) 616-620.

[8] Brunnemann K.D., Hoffmann D., Decreased concentrations of N-nitrosodiethanolamine and N-nitrosomorpholine in commercial tobacco products, *Journal of agricultural and food chemistry*, 39 (1) (1991) 207-208.

[9] Schmidtsdorff S., Neumann J., Schmidt A.H., Parr M.K., Prevalence of nitrosamine contaminants in drug samples: Has the crisis been overcome?, *Archiv der Pharmazie*, 356 (2) (2023) 2200484.

[10] Steinberg P., Klingelhöffer A., Schäfer A., Wüst G., Weisse G., Oesch F., Eigenbrodt E., Expression of pyruvate kinase M 2 in preneoplastic hepatic foci of N-nitrosomorpholine-treated rats, *Virchows Archiv*, 434 (1999) 213-220.

[11] Reznik-Schüller H., Sequential morphologic alterations in the bronchial epithelium of Syrian golden hamsters during N-nitrosomorpholine-induced pulmonary tumorigenesis, *The American Journal of Pathology,* 89 (1) (1977) 59.

[12] Gezer E., Özer C., Şimşek T., Yaprak Bayrak B., Turan G., Çetinarslan B., ... & Köksalan D., N-Nitrosomorpholine-induced oncocytic transformation in rat endocrine organs, *European Journal of Medical Research,* 29 (1) (2024) 64.

[13] Kostić D.A., Dimitrijević D.S., Stojanović G.S., Palić I.R., Đorđević A.S., Ickovski J.D., Xanthine oxidase: isolation, assays of activity, and inhibition, *Journal of chemistry*, 2015 (1) (2015) 294858.

[14] Hille R., Xanthine oxidase—a personal history, *Molecules,* 28 (4) (2023) 1921.

[15] Ullah Z., Yue P., Mao G., Zhang M., Liu P., Wu X., ... & Yang L., A comprehensive review on recent xanthine oxidase inhibitors of dietary based bioactive substances for the treatment of hyperuricemia and gout: Molecular mechanisms and perspective, *International Journal of Biological Macromolecules*, (2024) 134832.

[16] Massey V., Brumby P E., Komai H., Palmer G., Studies on milk xanthine oxidase: some spectral and kinetic properties, *Journal of Biological Chemistry*, 244 (7) (1969) 1682-1691.

[17] Masui T., Nakanishi H., Inada K.I., Imai T., Mizoguchi Y., Yada H., ... & Tatematsu M., Highly metastatic hepatocellular carcinomas induced in male F344 rats treated with N-nitrosomorpholine in combination with other hepatocarcinogens show a high incidence of p53 gene mutations along with altered mRNA expression of tumor-related genes, *Cancer letters,* 112 (1) (1997) 33-45.

[18] Alston T.A., Porter D.J., Bright H.J., Enzyme inhibition by nitro and nitroso compounds, *Accounts of Chemical Research,* 16 (11) (1983) 418-424.

[19] Singh A., Singh K., Sharma A., Kaur K., Chadha R., Bedi P.M.S., Past, present and future of xanthine oxidase inhibitors: design strategies, structural and pharmacological insights, patents and clinical trials, *RSC Medicinal Chemistry,* 14 (11) (2023) 2155-2191.

[20] Yu D., Du J., He P., Wang N., Li L., Liu Y., ... & Li Y., Identification of natural xanthine oxidase inhibitors: Virtual screening, anti-xanthine oxidase activity, and interaction mechanism, *International Journal of Biological Macromolecules,* 259 (2024) 129286.

[21] Onat T., Emerk K., Sözmen E., İnsan biyokimyası, Palme yayıncılık, (2002) 208.