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1-Methyl-3-nitropyridine: An Efficient Oxidant of NADH in Non-enzymatic and Enzyme-mediated Processes

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It is shown that NADH can be effectively oxidized by 1-methyl-3-nitropyridine in non-enzymatic and enzyme-mediated processes. Mechanistic issues of these reactions are discussed. These processes seem to contribute to the observed cytotoxicity of 1-methyl-3-nitropyridine. A key role of 1-methyl-3-nitropyridinyl radical formed in the enzyme-mediated processes is emphasized.

Keywords: NADH; Xanthine oxidase; Pyridinium salts; Enzyme mediated processes

INTRODUCTION

Nicotinamide adenine dinucleotide, present in the cells in the reduced (NADH) and oxidized (NAD^+) forms, plays an important role in cellular metabolism.[1] Absolute concentrations of NADH and NAD^+ as well as their relative concentrations may affect the rates of NADH- or NAD^+-dependent reactions as the NADH/NAD^+ couple constitutes a driving force for these reactions. Thus, introducing into the cell an agent capable of shifting NADH ↔ NAD^+ equilibrium can disturb the cell function. The cells with impaired metabolism should be more sensitive to cytotoxic agents and in extreme cases severe metabolic dysfunction may lead to cellular death. Strategies to control cell growth by affecting NADH level and the ensuing ATP depletion have already been presented in the literature.[2–5]

It is well known that pyridinium salts can reversibly react with NADH through the hydride anion transfer.[6–12] Equilibrium of this reaction depends on redox properties of the salt and therefore on substituent effect. Investigation of the close NAD^+ analogues, 1-methylpyridinium salts carrying various substituents at 3 position indeed confirmed their potency of shifting NADH ↔ NAD^+ equilibrium. Moreover, the equilibrium constant for the reaction of NADH with pyridinium salts could be correlated with their cytotoxic properties against murine leukemia L1210 cell culture.[13]

This paper describes for the first time a use of 1-methyl-3-nitropyridinium salt (**MNP**$^+$) as a unique agent effectively shifting NADH ↔ NAD^+ equilibrium. In contrast to other pyridinium salts **MNP**$^+$ can affect this equilibrium both in non-enzymatic and enzyme-mediated processes, therefore becoming a very powerful cytotoxic agent. Mechanisms concerning the above processes will be elucidated.

MATERIALS AND METHODS

Materials

Xanthine oxidase (Grade 1: from buttermilk, EC 1.1.3.22.), cytochrome c reductase (NADH dehydrogenase, from porcine heart crude, EC 1.6.99.3), superoxide dismutase (from bovine erythrocytes, EC 1.15.1.1.), cytochrome c (from horse heart), partially acetylated cytochrome c (from horse heart), β-NADH and other chemicals were obtained

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from Sigma-Aldrich, and were of highest available purity. Deionized, double distilled water (conductivity 0.1 μS/cm) was used.

Preparation of 1-Methyl-3-nitropyridinium Chloride

A solution of 3-nitropyridine (prepared according to the described procedure,[14] 30 mmol) and methyl iodide (Fluka, 60 mmol) in 20 ml of distilled methanol was kept at room temperature for 10 days. The precipitated 1-methyl-3-nitropyridinium iodide was filtered off, washed with methanol, dried and then converted to chloride by shaking its water solution with freshly precipitated silver chloride. Mp. 187–188°C (dec).

The same procedure was applied for the preparation of 1-[^{14}C]methyl-3-nitropyridinium chloride (3.2 μmol, 0.557 MBq/μmol), starting from [^{14}C]methyl iodide (10 μmol, 5.66 MBq) and 3-nitropyridine (5 μmol).

Preparation of 1-Methyl-3-nitro-1,4-dihydropyridine

To a stirred solution of **MNP**$^+$Cl$^-$ (7 mmol) in 20 ml of water a solution of 1-methyl-1,4-dihydronicotinamide (5 mmol) in 10 ml of water was added over a period of 10 min. The resulting dark-red solution was extracted with chloroform (3 × 50 ml), the extract dried over $MgSO_4$ and the solvent evaporated under the reduced pressure. The remaining red solid was purified by column chromatography (silica gel G, chloroform) to give light-red crystals, mp. 112–114°C.

^{1}H NMR (Bruker 250 MHz, $CDCl_3$) δ ppm: 3.14 (s, 3H, CH_3), 3.45 (s, 2H, CH_2), 5.24 (m, 1H), 5.75 (m, 1H), 7.79 (s, 1H).

Kinetic Measurements

Rates of reactions were measured spectrophotometrically in aqueous solutions by monitoring the decay of the absorption of NADH and growth of the absorption of 1-methyl-3-nitro-1,4-dihydropyridine (**MNPH**). Reacting solutions were thermostated in a cell holder at 25°C, shielded from light. UV-VIS absorption spectra were recorded on a Perkin Elmer Lambda 40 spectrophotometer. The pH of the solutions was adjusted with perchloric acid, sodium hydroxide and/or phosphate buffer and measured with an ORION 420A pH meter (Orion Research, Inc.).

When needed the solution was deoxygenated by bubbling with nitrogen for at least 10 min in the cell sealed with septum just before measurement.

To test the incorporation of hydrogen from water to the products during the reaction between NADH and **MNP**$^+$, the reaction was also carried out in the solution of tritiated water (72.8 MBq/g). Samples of **MNPH** were separated by $CHCl_3$ extraction. After removing of $CHCl_3$ and traces of water, the radioactivity of **MNPH** was determined by the liquid scintillation method.

Pulse Radiolysis Studies

Pulse radiolysis experiments were carried out with high energy (6 MeV) electron pulses (2 or 17 ns) generated from ELU-6 linear electron accelerator. The dose delivered per pulse was within the range 2–40 Gy. Details of pulse radiolysis system are given elsewhere.[15,16]

The pulse radiolysis of neutral water produces three highly reactive species: e_{aq} (2.6), $^\bullet$OH (2.7), H$^\bullet$ (0.6) in addition to the formation of less reactive products: H_2O_2 (0.7), H_2 (0.45), H_3O^+ (2.6) (numbers in parentheses represent the yield of radicals per 100 eV of energy absorbed).[17]

Reduction of **MNP**$^+$ was carried out in aqueous solution containing 0.1 M HCOONa, 5 mM phosphate buffer, pH 5.9 and 2 mM **MNP**$^+$Cl$^-$. In such system the electrons are scavenged by **MNP**$^+$ (Reaction 1) and hydroxyl radicals and hydrogen atoms are converted to carbon dioxide radical anion (Reactions 2 and 3), which is also a reducing agent (Reaction 4).

$$MNP^+ + e_{aq} \rightarrow MNP^\bullet \quad (1)$$

$$HCOO^- + {}^\bullet OH \rightarrow CO_2^{\bullet -} + H_2O \quad (2)$$

$$HCOO^- + H^\bullet \rightarrow CO_2^{\bullet -} + H_2 \quad (3)$$

$$MNP^+ + CO_2^{\bullet -} \rightarrow MNP^\bullet + CO_2 \quad (4)$$

The rate constant for the reaction of 1-methyl-3-nitropyridinyl radical (**MNP**$^\bullet$) with cytochrome c^{3+} (Reaction 5) was measured in deoxygenated solution containing HCOONa (50 mM), **MNP**$^+$Cl$^-$ (2 mM), phosphate buffer pH 7.8 (50 mM), EDTA (1 mM) and cytochrome c^{3+} (10–50 μM) based on kinetic analysis of the product (cytochrome c^{2+}, $\lambda_{max} = 550$ nm) build-up.

$$\text{cytochrome c}^{3+} + MNP^\bullet \rightarrow \text{cytochrome c}^{2+} + MNP^+ \quad (5)$$

Enzymatic Assay

The xanthine oxidase activity with NADH as a reducing substrate was measured spectrophotometrically by analysis of the decay of NADH absorption at 340 nm or cytochrome c^{3+} (cyt^{3+}, 50 μM) reduction on the base of the absorbance build-up at 550 nm. The reaction was run in solution containing 0.2 U/ml xanthine oxidase, 300 μM

NADH, 50 mM phosphate buffer (pH = 7.8), 1 mM EDTA, T = 25°C. Rate of enzymatic reaction was measured against the reference solution containing no enzyme.

The NADH dehydrogenase (cytochrome c reductase, 1 U/ml) activity in the presence of **MNP**$^+$ was measured analogously as for xanthine oxidase, except that the acetylated cytochrome c instead of cytochrome c was used.

Cytotoxic Assay

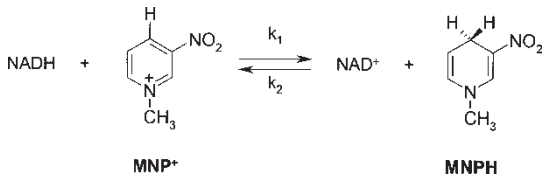
L1210 leukemia cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (Gibco) at 37°C in 5% CO_2 atmosphere. Cells were seeded in 24 well plates ($2{\cdot}10^4$ cells/well) and treated for 72 h with **MNP**$^+$ in triplicates in a final volume of 2 ml. Cells were counted in Fuchs-Rosenthal hemacytometer.

1-Methyl-3-nitropyridinium Chloride Uptake by L1210 Cells

The 10^7 of L1210 cells were suspended in 1.8 ml of RPMI 1640 medium and 0.2 ml of the solution of 2.0 mM ^{14}C-**MNP**$^+$ (7.5 mCi/ml) was added. The cells were incubated at 37°C in 5% CO_2 atmosphere. Every hour samples of 0.2 ml were taken, diluted in 10 ml of cold phosphate-buffered saline (PBS) and centrifuged for 5 min. The sediment was resuspended and lysed. After 24 h of incubation at 37°C the contents were quantitatively transferred to scintillation vials. The radioactivity was determined by application of liquid scintillation technique using Triton X-100.

RESULTS AND DISCUSSION

It has been observed that NADH reacts reversibly with **MNP**$^+$ in water according to reaction 6. The reaction is easily followed by monitoring NADH decay at 340 nm and **MNPH** formation at 450 nm as shown in Fig. 1.



(6)

The rate constants for both forward ($k_1 = 3.5{\cdot}10^{-1}\,M^{-1}s^{-1}$) and reverse ($k_2 = 1{\cdot}10^{-5}\,M^{-1}s^{-1}$) reactions as well as equilibrium constant ($K = 3.5{\cdot}10^4$) were found. It is concluded based on numerous literature studies[6–12] involving NADH analogs and substituted pyridinium or quinolinium salts and our studies[13] concerning the reactions of NADH with pyridinium salts carrying various substituents that the reaction 6 involves one step hydride transfer.

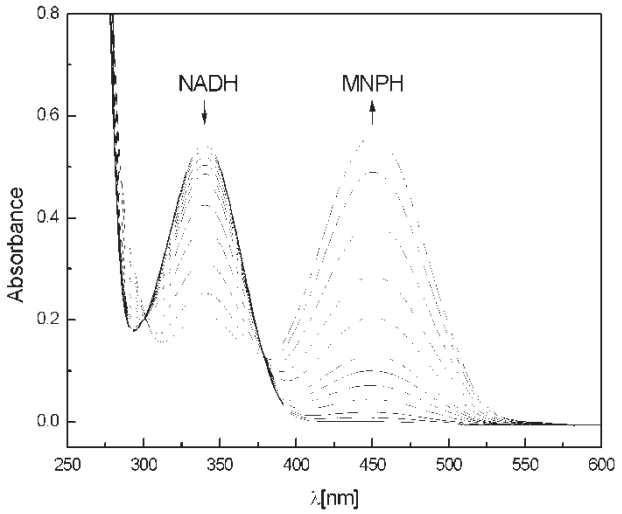


FIGURE 1 Changes in the electronic absorption spectra of equimolar (0.1 mM) mixture of NADH and **MNP**$^+$ in aqueous solution. Presented time scale of 10 h. T = 25°C, l = 1.0 cm.

Similarly to our former studies[18–21] concerning characterization of the transient radical species generated from NADH analogues we have been able to characterize the radical (**MNP**$^\bullet$) formed by one-electron reduction of **MNP**$^+$ (see Fig. 2), however, an evidence for participation of this species as intermediate in the reaction 6 was not found. First of all, in the reaction of NADH with **MNP**$^+$ in the presence of cyt c^{3+} (50 μM) no reduction of cytochrome c was spectroscopically observed. The reaction of **MNP**$^\bullet$ with cyt c^{3+} is fast enough ($k = 1.9{\cdot}10^6\,M^{-1}s^{-1}$) to effectively compete under pulse radiolysis experimental conditions with other reactions leading to **MNP**$^\bullet$ decay (for example, the reaction with oxygen or radical recombination). Additionally during the reaction of NADH with **MNP**$^+$ there was no incorporation of tritium from labeled water into the product-**MNPH**.

It is known that NADH can be a substrate for the number of flavoenzymes as, for example, for xanthine oxidase (XO).[22,23] XO-mediated oxidation of NADH to NAD$^+$ results also in the formation of superoxide radical anion ($O_2^{\bullet-}$). This process can be activated by a number of easily reducible agents, which catalyze formation of the excessive amount of $O_2^{\bullet-}$.[22–25]

It is shown in Fig. 3 that **MNP**$^+$ can activate oxidation of NADH mediated by XO. The K_m = 180 μM was estimated for this reaction in the presence of oxygen at fixed concentration of NADH (300 μM). The presence or absence of oxygen did not affect the enzymatic and non-enzymatic reaction rates.

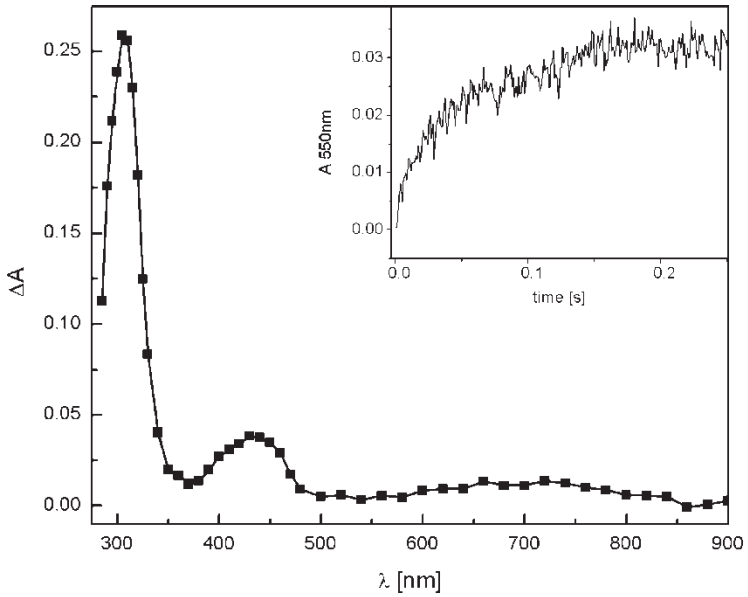


FIGURE 2 Transient absorption spectrum of **MNP**$^{\bullet}$ radical obtained on reduction of **MNP**$^{+}$ (2 mM) by pulse radiolysis of aqueous solution. Dose per pulse 32 Gy. Insert: Build-up of 550 nm absorption of the cyt c^{2+} reduced in reaction with **MNP**$^{\bullet}$ (see text). Dose 4 Gy.

NADH interacting with flavin center of XO causes its reduction and **MNP**$^{+}$ reoxidizes the enzyme (Scheme 1). The formation of **MNP**$^{\bullet}$ radical in this reaction is confirmed by one-electron reduction of cyt c^{3+}. The rate of cytochrome c reduction is again not affected by oxygen and almost doubles NADH oxidation rate. This indicates that **MNP**$^{+}$ is the only oxidizing agent in the enzymatic reaction. The experiments conducted in the presence of superoxide dismutase (SOD, 40 U/ml) exclude the formation of $O_2^{\bullet-}$ in the enzymatic reaction.

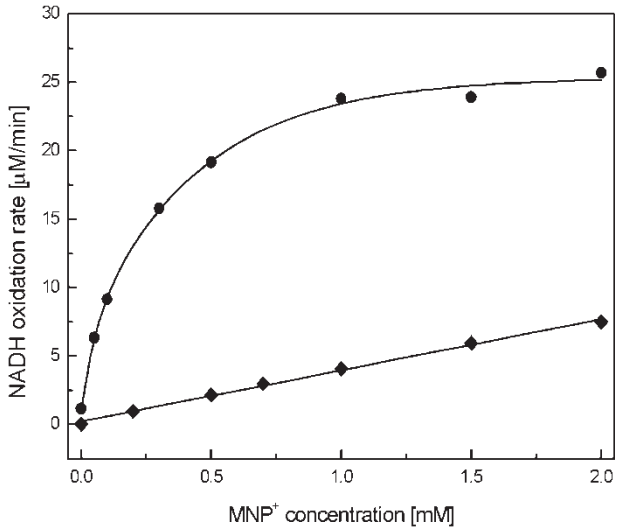
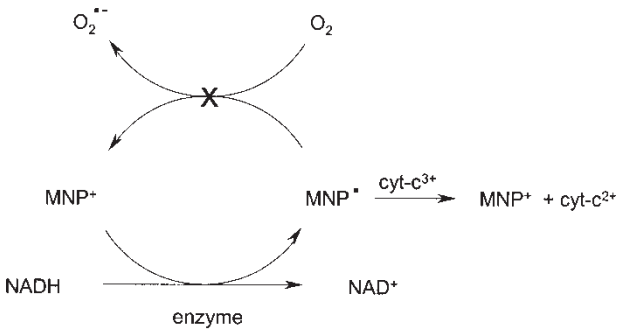


FIGURE 3 Rates of **MNP**$^{+}$ (0–2 mM) activated enzymatic (●) and non-enzymatic (◆) oxidation of NADH (0.3 mM).

We have also observed similar processes of reductive activation of **MNP**$^{+}$ for NADH dehydrogenase (cytochrome c reductase).

Based on the evidence presented above that **MNP**$^{+}$ can effectively oxidize NADH in non-enzymatic or enzyme-mediated processes with formation of radicals one can expect that **MNP**$^{+}$ can also function as the cytotoxic agent. Indeed, it was found that **MNP**$^{+}$ possesses remarkable cytotoxic activity against cultured murine leukemia L1210 cells. In terms of ED_{50} (the drug concentration effective in inhibiting 50% of the cell growth after 72 h exposure of L1210 cells to the drug) **MNP**$^{+}$ was found to be very active antiproliferative agent ($ED_{50} = 3\,\mu M$) with the activity comparable to that of antitumour drug cisplatin.[26] It is conceivable that formation of radicals in the enzyme-mediated processes activated by **MNP**$^{+}$ is mostly responsible for the cytotoxicity observed. The accumulation and relatively high transport rate



SCHEME 1

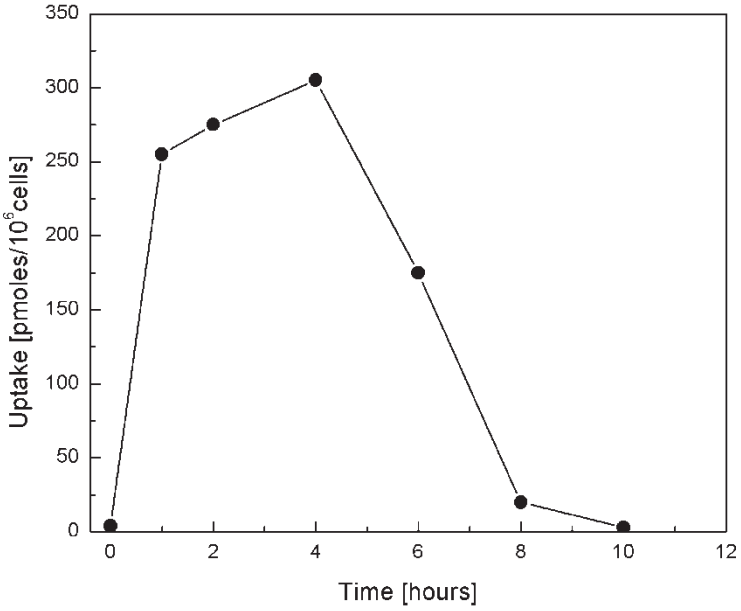


FIGURE 4 Kinetics of **MNP**$^+$ uptake by L1210 cells. Cells at the density of 5×10^6/ml were incubated in a growth medium with 100 μM ^{14}C-**MNP**$^+$ at 37°C. 2 ml aliquots of cell suspension was taken at indicated intervals and the amount of the radioactive drug accumulated by L1210 cells was measured.

of ^{14}C-**MNP**$^+$ into L1210 cells presented in Fig. 4 indicates that despite of its ionic character **MNP**$^+$ is able to overcome transportation barriers through the plasma membranes and therefore its intracellular concentration might be sufficient to disturb the cell metabolism. After 1 h of cells treatment the accumulation of ^{14}C-**MNP**$^+$ reaches the level of approximately 260 pmoles per 10^6 cells. The rapid decrease of **MNP**$^+$ accumulation in L1210 cells observed after 4 h of treatment (Fig. 4) was caused by disintegration of the cells due to a excessive drug action. This may point toward alternative mechanism of **MNP**$^+$ cytotoxicity that this compound can interact extracellularly with flavoenzymes presented in the cell membranes and the generated radicals damage the membranes leading to the cell death. Moreover, partly damaged cell membranes might allow for a less restricted **MNP**$^+$ penetration through the membranes.

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